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Host morphogenetic events and Wnt signaling influence Wolbachia tropism in Drosophila gonads

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HOST MORPHOGENETIC EVENTS AND WNT SIGNALING INFLUENCE *WOLBACHIA* TROPISM IN *DROSOPHILA* GONADS

by

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ABSTRACT

Wolbachia are maternally transmitted endosymbionts that infect a large proportion of arthropods and reduce vector competency of mosquitoes carrying deadly diseases such as Dengue, Zika, West Nile Virus, and Chikungunya. *Wolbachia* preferentially infect specific host cells, a phenomenon termed tissue tropism. *Wolbachia* tissue tropism has consequences in proper vertical and horizontal transmission. Although *Wolbachia* tropism to multiple *Drosophila* gonadal cell types has been identified, the mechanisms of tropism during development have not been characterized, in part because *Wolbachia* infected cell types are challenging to study during morphogenesis. Here we describe a novel *Wolbachia* tropism to polar cells (PCs) of the *Drosophila* ovary, a developmentally well characterized system. We show that *Wolbachia* intracellular accumulation is triggered by specific events of PC morphogenesis including differentiation from progenitors, and during stage 8 to 9 transition. Using genetic tools, we also demonstrate that induction of ectopic PC fate is sufficient to drive *Wolbachia* tropism. These findings implicate a tight coordination of host developmental events with *Wolbachia* tropism.

Wolbachia tropism to multiple host cell types suggest that host pathways common to these would be conducive to intracellular *Wolbachia* growth. Indeed, we found that Wnt signaling, is active in gonadal cell types with *Wolbachia* tropism. Wnt signaling, first characterized in embryonic development and patterning, has novel functions in immunity and intracellular pathogen survival. Using RNAi mediated gene knockdowns, we studied the effect of Wnt signaling on *Wolbachia* in various infected cell types, including the testis stem cell niche, PCs and germline. Reduction of Wnt signaling caused a decrease in *Wolbachia* density and increased signaling led to higher density suggesting the reliance of *Wolbachia* on host Wnt signaling for its tropism. Moreover, expression of ectopic Wnt signaling was sufficient to drive *Wolbachia* tropism to previously uninfected tissues such as the *D. melanogaster* female germline stem cell niche. Finally, small molecule Wnt signaling agonists were sufficient to drive high *Wolbachia* titers in mosquito cell lines. These findings describe the effect of host signaling on *Wolbachia* tropism and provide an approach to affect *Wolbachia* levels in disease-causing vectors, thereby contributing to *Wolbachia* based vector control strategies.

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LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
Arr	Arrow
BDSC	Bloomington *Drosophila* Stock Center
Dsh	Disheveled
DSHB	Developmental Studies Hybridoma Bank
FCs	Follicle cells
Fz	Frizzled
GSCN	Germline Stem Cell Niche
NGS	normal goat serum
Pan	Pangolin
PBANG	phosphate buffered saline with Triton-X 100, bovine serum albumin and normal goat serum
PBS	phosphate buffered saline
PBS/BSA	phosphate buffered saline with bovine serum albumin
PBT	phosphate buffered saline with Triton-X 100
PCs	Polar Cells
PCR	polymerase chain reaction
PFA	paraformaldehyde
qPCR	Quantitative PCR
qRT-PCR	Quantitative Real Time PCR

RNAi	RNA interference
SCs	Stalk cells
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Sgg/GSK3	Shaggy/Glycogen Synthase Kinase 3
SSC	Somatic Stem Cell
SSCN	Somatic Stem Cell Niche
Tris	tris(hydroxymethyl)aminomethane
UAS	Upstream Activating Sequence
Upd	Unpaired
Wg	Wingless

CHAPTER 1 INTRODUCTION

1.1 Bacteria and multicellular organisms

Most multicellular organisms are colonized by diverse populations of microorganisms. The metazoan and its bacterial partners have evolved co-dependency on each other. The microbes shape the metazoan host by affecting their evolution, nutrition, physiology, and development (Fraune and Bosch 2010, Lee and Brey 2013, McFall-Ngai *et al.* 2013). Insects being the most abundant class of organisms on the planet (Stork 1988, Basset *et al.* 2012), provide many examples of such interactions (Engel and Moran 2013, Lee and Brey 2013). For instance, in absence of *Lactobacillus* and *Acetobacter*, *Drosophila* larvae fail to develop on a nutrient poor diet (Shin *et al.* 2011, Storelli *et al.* 2011). Presence of live aerobic bacteria is essential for successful development of mosquitoes *Aedes aegypti* (Coon *et al.* 2014). Some of these microbial partners eventually evolve to be obligatory for insect survival and become maternally transmitted. For example, *Buchnera*, symbionts of aphids, synthesize essential amino acids required for normal development and reproduction of the host (Shigenobu *et al.* 2000, Koga *et al.* 2007, Hansen and Moran 2011). Moreover, Tsetse flies rely on their symbiont *Wigglesworthia* for reproduction, digestion, longevity and immunity (Pais *et al.* 2008).

Many insects that harbor bacterial symbionts are vectors of devastating human diseases such as malaria, dengue, zika, west nile virus, chikungunya, and trypanosomiasis among others. Apart from playing an important role in many aspects of host biology, some symbionts help to augment the insects' defenses against viruses and parasites (Weiss and Aksoy 2011, Jupatanakul *et al.* 2014). This has led to the development of novel ways to

control the vector competency, the ability to transmit human diseases, of these insects (Durvasula *et al.* 1999, Rio *et al.* 2004, Weiss and Aksoy 2011). For instance, Gram negative midgut bacteria inhibited the growth of *Plasmodium* parasites in mosquitoes (Pumpuni *et al.* 1993, Dong *et al.* 2009). Certain *Chromobacterium spp*. can reduce Dengue virus in *Aedes aegypti* mosquito upon midgut infection (Ramirez *et al.* 2014). In Tsetse flies, the symbiont *Wigglesworthia* inhibits *Trypanosome* parasite growth (Pais *et al.* 2008, Weiss and Aksoy 2011). In most cases, bacterial symbionts confer resistance to viruses and parasites by enhancing the host immunity.

This phenotype of resistance against viruses is most widespread and pronounced upon infection by *Wolbachia pipientis,* a maternally transmitted endosymbiont from the *Rickettsiales* order that infects a significant proportion of insects (Hedges *et al.* 2008, Walker *et al.* 2011).

1.2 *Wolbachia* are maternally transmitted obligate endosymbionts with potential to control vector transmitted diseases

Wolbachia are maternally transmitted obligate endosymbionts of the order *Rickettsiales*. They infect between 40-70% of invertebrates (Hilgenboecker *et al.* 2008, Zug and Hammerstein 2012). To facilitate their transmission, *Wolbachia* induce a variety of reproductive phenotypes in the host that usually improve the reproductive fitness of the infected females (Werren *et al.* 2008, Fast *et al.* 2011). Moreover, they also significantly affect host lifespan (Min and Benzer 1997, Chrostek and Teixeira 2015), fecundity (Fast *et al.* 2011), and immunity (Hedges *et al.* 2008, Osborne *et al.* 2009, Bian *et al.* 2010, Kambris *et al.* 2010). *Wolbachia* increase the host viability by providing broad resistance

against viruses such as *Drosophila* C virus and Flock House Virus in *Drosophila* species (Hedges *et al.* 2008, Osborne *et al.* 2009, Martinez *et al.* 2014). This effect is also found in mosquitoes and is used to control the spread of vector transmitted diseases like Dengue (Moreira *et al.* 2009, Bian *et al.* 2010), Chikungunya (Blagrove *et al.* 2013), Plasmodium (Moreira *et al.* 2009, Bian *et al.* 2013) and more recently Zika virus (Aliota *et al.* 2016, Caragata *et al.* 2016, Schultz *et al.* 2017).

These features make *Wolbachia* an excellent tool for vector-transmitted disease control. However, the mechanisms of *Wolbachia* mediated antiviral response are not completely understood. In many cases, symbiont-antiviral response is due to enhanced immunity. For instance, *Chromobacterium* infection of *Ae. aegypti* midguts activates the immune response against both *Plasmodium* and Dengue virus (Ramirez *et al.* 2014). The midgut bacteria in *Ae. aegypti* affect Dengue virus via the Toll pathway, an immune response pathway (Xi *et al.* 2008). However, it has been shown that *Wolbachia* can inhibit viruses even without activating the host immune response (Bourtzis *et al.* 2000, Rances *et al.* 2012, Chrostek *et al.* 2014). Resource competition between *Wolbachia* and the viruses is a possible mechanism for antiviral effects of *Wolbachia*. Lipids, for instance, are essential for arboviruses such as Dengue virus and West Nile virus to infect and proliferate in both vertebrate and insect hosts (Chu and Ng 2004, Chu *et al.* 2006, van der Schaar *et al.* 2007, Martin-Acebes *et al.* 2011). *Wolbachia* also uses lipids and competes with the viruses for limited resources (Moreira *et al.* 2009, Caragata *et al.* 2013, Sinkins 2013). Recently Schultz *et al.* showed that Zika virus growth can be rescued by adding dietary cholesterol in *Wolbachia* infected cell lines (Schultz *et al.* 2017). Due to resource

competition, virus exclusion has been observed in *Wolbachia* infected cells in *Ae. aegypti* mosquitoes (Moreira *et al.* 2009).

Studies suggest that there is a correlation between *Wolbachia* intracellular densities and viral inhibition. Higher *Wolbachia* densities lead to a better protection against viruses whereas low *Wolbachia* loads lead to inefficient viral blockage (Osborne *et al.* 2009, Frentiu *et al.* 2010, Lu *et al.* 2012, Osborne *et al.* 2012, Bian *et al.* 2013, Schultz *et al.* 2017). Thus, understanding the molecular mechanisms of *Wolbachia* intracellular accumulation and tissue tropism, specific colonization of bacteria to specific cell types, are important to better utilize *Wolbachia* biology as a novel method for vector transmitted disease control. *Drosophila melanogaster*, the fruit fly, is a powerful model organism to answer these questions. With readily available genetic tools, molecular markers, short generation time, and multiple *Wolbachia* endosymbionts, it is an excellent model system to study insect-*Wolbachia* interactions (Ejsmont and Hassan 2014). *Wolbachia* show high levels of tropism to specific cell types in the gonads which are described below.

1.3 *Wolbachia* tropism to the *Drosophila* gonads

1.3.1 Ovary

Each female *Drosophila* has 2 ovaries (Fig. 1.1A,B). A *Drosophila* ovary consists of 14-16 strings of developing egg chambers called ovarioles (Fig. 1.1B,C). At the anterior tip resides the germarium, a structure that harbors both the germline and somatic stem cells and their respective niches (Fig. 1.1C,D). The germline stem cell (GSC, red in Fig. 1.1D) divides asymmetrically and gives rise to an oocyte and 15 supporting nurse cells. The somatic stem cell (SSC, blue in Fig. 1.1D), located at the border region between regions 2a

and 2b, gives rise to transiently dividing follicle cells (FCs) (light blue in Fig. 1.1D). Each stem cell population resides in a specialized microenvironment, referred to as the stem cell niche. The GSC niche (GSCN) consists of the cap cells and terminal filament cells (green with bracket in Fig. 1.1D). The escort cells support the dividing germline cyst in regions 1 and 2a before they get encapsulated by the SSCN derived somatic cells. The most posterior escort cell (green in Fig. 1.1D) is generally considered to be the SSCN and it provides factors necessary for SSC maintenance and division (Sahai-Hernandez and Nystul 2013).

The germline cyst (Fig. 1.1D) consists of 16 cells: 1 oocyte and 15 supporting nurse cells. As it passes through the border region, it gets encapsulated by a layer of transiently dividing FCs. This cyst, now called an egg chamber (Fig. 1.1C,E), progresses through 14 stages of oogenesis maturing into an egg (King 1970, Spradling 1993). The FCs eventually differentiate into three distinct populations of cells: 1. The polar cells (PCs): two pairs of specialized cells at either pole of the egg chamber (red in Fig. 1.1E). 2. The lateral follicle cells (FCs): all the other follicle cells that encapsulate the egg chamber (dark gray in Fig. 1.1E). 3. The stalk cells (SC): a string of four to six stalk cells connect two adjacent egg chambers (Torres *et al.* 2003, Assa-Kunik *et al.* 2007). The PCs play an essential role in establishing the polarity and patterning of the egg chamber (Gonzalez-Reyes and St Johnston 1998, Grammont and Irvine 2002). PCs along with SCs are specified in the germarium and stop dividing soon after the egg chamber exits the germarium (Ruohola *et al.* 1991, Tworoger *et al.* 1999, Zhang and Kalderon 2000, Grammont and Irvine 2001). The lateral FCs continue to divide transiently and increase in number until stage 6 of oogenesis (Gonzalez-Reyes and St Johnston 1998). After stage 6, they grow in size by

undergoing endoreplication. It is possible to observe most developmental stages of these cells from stem cell division to egg maturation in a single ovary (Spradling 1993, Wu *et al.* 2008) making *Drosophila* oogenesis a powerful system to study *Wolbachia* tropism kinetics during development.

Being maternally transmitted, *Wolbachia* colonize the female germline at high densities. Apart from the germline, *Wolbachia* also infect certain somatic cell types at high densities. Previous research in the Frydman lab has characterized *Wolbachia* tropism to both the germline stem cell niche (GSCN) and somatic stem cell niche (SSCN) of *Drosophila* ovaries (Frydman *et al.* 2006, Toomey *et al.* 2013, Toomey and Frydman 2014). The SSCN tropism is conserved across all *Wolbachia* strains tested whereas the GSCN tropism is more variable (Toomey *et al.* 2013). Upon horizontal transmission, the SSCN is the first tissue to be colonized by *Wolbachia* (Frydman *et al.* 2006). Toomey *et al.* had previously proposed that SSCN *Wolbachia* infection facilitates high *Wolbachia* titers in the germline thereby assisting in maternal transmission (Toomey *et al.* 2013).

1.3.2 Testis

A *Drosophila* male has two testes that produce and secrete sperm (Fig. 1.2A,B). At the apical tip of the testis, the GSCs (dark gray cells in Fig. 1.2C) and cyst stem cells (CySCs, white crescent cells in Fig. 1.2C) reside next to their common niche, the hub (red cells in Fig. 1.2C). Previously, *Wolbachia* tropism to the hub was described by Toomey *et al*. (Toomey and Frydman 2014). As males are a dead end for *Wolbachia* in terms of vertical transmission, there is extreme divergence of *Wolbachia* tropism phenotypes to the hub across various *Wolbachia* strains (Toomey and Frydman 2014).

1.4 *Wolbachia*-host molecular interactions

The molecular mechanisms of *Wolbachia* tropism to specific host cells are not well characterized. Many examples of *Wolbachia* manipulating host biology to assist their own maternal transmission exist. In *Drosophila mauritiana (D.mau), Wolbachia* increase stem cell division and reduce programmed cell death to cause infected flies to lay four times more eggs (Fast *et al.* 2011). *Wolbachia* have been to manipulate the host Toll immune pathway to facilitate their own persistent infection. They upregulate the Toll pathway via reactive oxygen species (ROS) activation (Pan *et al.* 2012). Other studies have found that *Wolbachia* upregulate host metalloproteases in *Ae. aegypti* probably to assist in their invasion of host tissues. Knockdown of these metalloproteases led to a reduction in intracellular *Wolbachia* (Hussain *et al.* 2011). *Wolbachia* also cause hypomethylation of the host genome by downregulating DNA methyltransferases. Again, overexpression of the methyltransferase led to a *Wolbachia* depletion (Zhang *et al.* 2013). In both of these cases, *Wolbachia* were shown to modulate host microRNAs to affect transcript levels of the affected genes (Hussain *et al.* 2011, Zhang *et al.* 2013).

However, there are limited studies into which host signaling processes control *Wolbachia* tropism and density. An overview of some of these processes is presented below.

1.4.1 Host signaling pathways that affect *Wolbachia* growth

Being an obligate symbiont, *Wolbachia* encounter multiple host cells and immune defense mechanisms, which they must evade to grow and spread efficiently. Autophagy, a major intracellular immune response, is known to regulate *Wolbachia* titers in somatic

tissues of multiple hosts including *Drosophila*, the mosquito *Culex pipiens*, and the worm *Brugia malayi* (Voronin *et al.* 2012). Voronin *et al*. upregulated autophagy in somatic cell types by exposure to rapamycin, an inhibitor of TOR. In these cells, they observed a significant reduction of *Wolbachia* levels (Voronin *et al.* 2012). Moreover, activation of autophagy was found during periods of rapid *Wolbachia* growth. The authors proposed that *Wolbachia* levels are maintained by increased autophagy in these somatic cell types (Voronin *et al.* 2012). Contrary to this, Serbus *et al*. demonstrated that rapamycin exposure led to an increase in *Wolbachia* levels in *Drosophila* germline tissue (Serbus *et al.* 2015). These findings suggest a positive interaction between host autophagy and germline *Wolbachia* levels. These conflicting results indicate that host autophagy may regulate *Wolbachia* in a tissue-specific manner.

The host cytoskeleton has been implicated in proper maternal transmission of *Wolbachia*. Knockdown of *profilin* or *villin* led to decreased *Wolbachia* titers in the *Drosophila* egg (Newton *et al.* 2015). Both Profilin and Villin are required for the dynamic rearrangement of the actin cytoskeleton during oogenesis. Actin cytoskeleton may be essential for proper localization of *Wolbachia* in the oocyte and efficient transmission (Newton *et al.* 2015). These findings add to previous studies that implicate microtubules and *dynein* in proper *Wolbachia* localization in the *Drosophila* oocyte and efficient transmission (Ferree *et al.* 2005). The microtubule reorganization during oogenesis coincides with *Wolbachia* redistribution in the egg. The microtubules along with Dynein complex regulate the proper localization of *Wolbachia* to the oocyte, an essential part of maternal transmission. Blockage of microtube reorganization by using colchicine led to a

reduction in *Wolbachia* titers in the oocyte (Ferree *et al.* 2005). Both these studies together show that proper *Wolbachia* localization in the oocyte during development is key to their maternal transmission.

These studies implicate various host pathways in affecting *Wolbachia* intracellular densities in specific tissues of the host, primarily the germline. However, there is a lack of understanding of why *Wolbachia* infects only certain cell types and not others. Also, there are contrasting effects of pathways such as autophagy on *Wolbachia* in germline and somatic tissues (Voronin *et al.* 2012, Serbus *et al.* 2015). Other intracellular bacteria may provide us with clues to understanding the molecular mechanisms of *Wolbachia* tropism.

1.4.2 Microbes and Wnt pathway

Innate immunity mediated by the Wnt pathway has been implicated in phagocytosis of microbes and their intracellular survival (Silva-Garcia *et al.* 2014). One well-studied case is *Salmonella*, an intracellular bacterium which infects animal guts. Upon infection of intestinal cells, *Salmonella* activates Wnt signaling in intestinal epithelial cells and induces proliferation of these cells (Sun *et al.* 2004, Sun *et al.* 2005, Duan *et al.* 2007, Ye *et al.* 2007, Liu *et al.* 2010). *Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects alveolar macrophages in the lungs (Jayachandran *et al.* 2014). To escape lysis in the macrophagic phagosome, *M. tuberculosis* upregulates Wnt signaling which results in an inhibition of lysosome-phagosome fusion (Blumenthal *et al.* 2006, Maiti *et al.* 2012, Villasenor *et al.* 2017). Moreover, this upregulation of the Wnt pathway increases inflammation, leading to more *M. tuberculosis* entering macrophages (Villasenor *et al.* 2017). *Ehrlichia chaffeensis*, a close living relative of *Wolbachia*, is a human pathogen.

Recently, *Ehrlichia* has been shown to activate the Wnt pathway upon infection of its host (Luo *et al.* 2015, Mitra *et al.* 2018). It secretes factors that activate the Wnt pathway to facilitate cell entry and inhibit phagosome-lysosome fusion leading to better intracellular survival of the bacteria. (Luo *et al.* 2015, Mitra *et al.* 2018).

As *Wolbachia* also infect specific host cells and survive intracellularly, the interaction of *Wolbachia* and host Wnt signaling needs to be investigated.

1.4.3 A primer on the *Drosophila* Wnt pathway

The Wnt signaling pathway is a highly conserved, essential signaling pathway involved in development, morphogenesis, stem cell control, cell polarity, and cell fate specification (Siegfried and Perrimon 1994, DasGupta *et al.* 2005, Swarup and Verheyen 2012, Bejsovec 2013). Newer findings also implicate the role of Wnt pathway in immunity and bacterial intracellular survival (Staal and Clevers 2005, Staal *et al.* 2008, Zhu and Zhang 2013, Silva-Garcia *et al.* 2014, Villasenor *et al.* 2017). The Wnt pathway has three major branches, canonical Wnt signaling, Planar Polarity, and Wnt/Ca^{+2} signaling. Canonical Wnt signaling (Fig. 1.3) was the first pathway one to be discovered and characterized.

Wnt (*Drosophila* homolog: Wingless(Wg)), a secreted ligand binds to a transmembrane receptor, Frizzled (Fz) in the target cells and activates signaling. Arrow (Arr/LRP5), a coreceptor is required for activation of signaling. Successful binding of Wnt to Fz-Arr recruits the intracellular proteins to the membrane. Disheveled (Dsh/Dvl) binds to the cytosolic portion of Fz (Wong *et al.* 2003) and Axin, a component of the destruction complex binds to Arr (Mao *et al.* 2001). Armadillo (Arm, β-catenin), the signal transducer

of Wnt signaling, is bound to a destruction complex made of Axin, Shaggy/Glycogen synthase kinase 3 (Sgg/GSK3β) and Adenomatous polyposis coli (APC) (DasGupta *et al.* 2005, Bejsovec 2013, Stamos and Weis 2013). This complex marks Arm for ubiquitination and subsequent degradation by the proteasome (Stamos and Weis 2013). Upon Wnt signaling activation, the recruitment of Axin to LRP5 facilitated by Dsh leads to the inhibition of the destruction complex (Bejsovec 2013). The destruction complex is inhibition by phosphorylation of Sgg and possibly Axin (Tolwinski and Wieschaus 2004, Stamos and Weis 2013, Stamos *et al.* 2014). Upon inhibition of the destruction complex, Arm accumulates in the cytosol and translocates to the nucleus. Here, it binds to a co-transcription factor TCF/Pangolin (Pan) to activate transcription (Schweizer *et al.* 2003). In the absence of Arm, Pan is a transcriptional repressor (Cavallo *et al.* 1998, Song *et al.* 2010). This provides extreme cell type specificity to Wnt signaling (Bejsovec 2013).

1.6 Dissertation Rationale and Summary

Wolbachia biology is emerging as a novel method of controlling vector transmitted diseases like Dengue, Chikungunya, West Nile Virus, and Zika Virus (Hedges *et al.* 2008, Moreira *et al.* 2009, Bian *et al.* 2010, Aliota *et al.* 2016, Caragata *et al.* 2016, Schultz *et al.* 2017).

However, studies show that *Wolbachia* inhibition of viruses is not universal and is highly dependent on intracellular bacteria density (Osborne *et al.* 2009, Frentiu *et al.* 2010, Lu *et al.* 2012, Osborne *et al.* 2012, Schultz *et al.* 2017). In certain cases, such as *Anopheles stephensi*, *Wolbachia* infection is transient and is lost over time (Bian *et al.* 2013). This

highlights the need to understand *Wolbachia* tropism kinetics and host pathways affecting *Wolbachia* tropism to specific cell types.

Wolbachia tropism to the somatic cell types, including the stem cell niches, is essential for their transmission via the female germline. In chapter 3, we investigate the kinetics of *Wolbachia* tropism to a novel somatic cell type, the polar cells (PCs) of the *Drosophila* ovary. Previous observations of somatic *Wolbachia* tropism were in differentiated cells which are difficult to study during their morphogenesis. *Wolbachia* SSCN tropism (Frydman *et al.* 2006) can be observed only in adults. SSCN morphogenesis occurs during pupal development from a non-dedicated population of cells making it impossible to trace their lineages (Nystul and Spradling 2007, Sahai-Hernandez and Nystul 2013, Vlachos *et al.* 2015). Hub specification and morphogenesis occurs in mid embryogenesis (Le Bras and Van Doren 2006, Sheng *et al.* 2009) and its development spans multiple life stages of the insect making it harder to study *Wolbachia* tropism during development. *Wolbachia* PC tropism as described in chapter 3 provides a new powerful model to study *Wolbachia* tropism to specific cell types during morphogenesis and development, as one can observe all stages of PC development in a single ovariole. Our data suggest that *Wolbachia* coordinate their replication and accumulation to certain key host developmental events.

Further in chapter 4, we investigate the role of Wnt signaling in *Wolbachia* tropism. The presence of Wnt signaling in tissues with *Wolbachia* tropism along with the role of Wnt signaling in promoting the survival of intracellular bacteria prompted us to investigate the effect of this pathway on *Wolbachia*. Using the hub, a molecularly and cellularly well-

characterized structure, we investigated the role of Wnt signaling in facilitating *Wolbachia* intracellular growth. Moreover, we extend these findings to the PCs and germline in the ovary as well as mosquito cell lines, suggesting a conserved role of this pathway in facilitating *Wolbachia* tropism and high intracellular accumulation.

Figure 1.1 The *Drosophila* female ovary

(A) Diagram of a female *Drosophila* with the approximate location of the ovary. (B) Diagram of the ovaries. Each ovary has multiple strings of developing egg chambers called ovarioles. (C) Diagram of an ovariole. Development proceeds from left (anterior) to right (posterior). At the anterior tip of the ovariole is the germarium which houses the stem cells and their respective niches. The egg chambers arising from the germarium progress through 14 developmental stages to mature into an egg. (D) Diagram of a germarium. The GSCs (red) are anchored to their niche (GSCN, green with bracket). These divide to form 15 nurse cells and an oocyte, together called a germline cyst, which forms the germline (pink). The somatic stem cells (SSCs, dark blue) are anchored to their niche (SSCN, green). These divide to form the follicle cells (light blue) that enclose the germline cyst and support the developing egg chamber. (E) Diagram of an egg chamber. The developing egg chamber consists of the germline cells in the middle (light gray) surrounded by a layer of somatic follicle cells (dark gray). Two pairs of somatic cells at either pole are called the polar cells (PCs). These cells are defined early in oogenesis and control the polarity of the egg chamber.

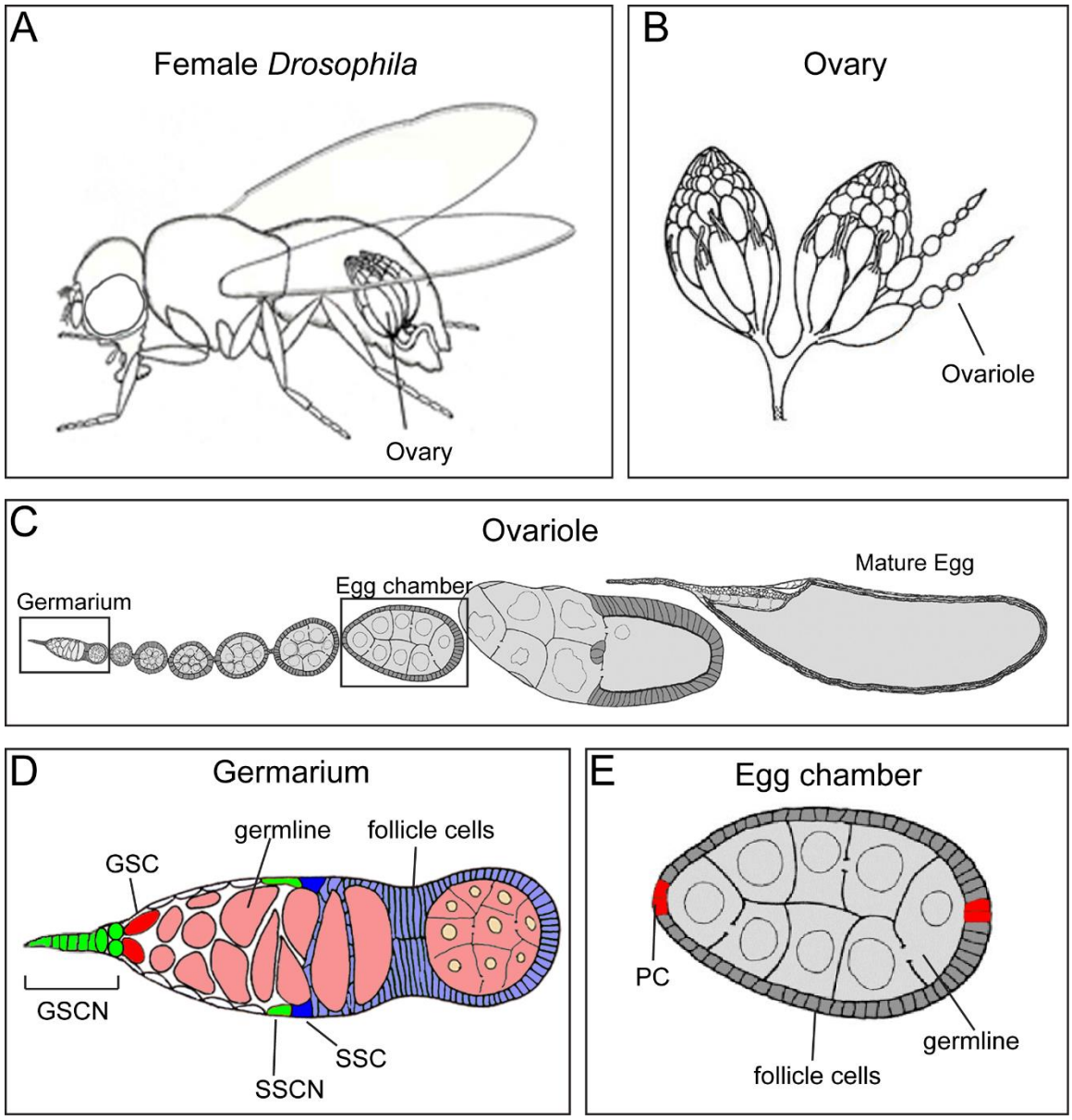

A
Female *Drosophila*
Ovary
B
Ovary
Ovariole
C
Ovariole
Germarium
Egg chamber
Mature Egg
D
Germarium
germline
follicle cells
GSC
GSCN
SSC
SSCN
E
Egg chamber
PC
germline
follicle cells

Figure 1.2 The *Drosophila* male testis

(A) Diagram of a male *Drosophila* showing the approximate location of the testis. (B) Diagram of the testis with the apical tip marked in a square. (C) Diagram of the apical tip of the testis. Each germline stem cell (GSC, gray) is surrounded by two crescent shaped cyst stem cells (CySCs) and are radially arranged around the hub (red bordered cells). The GSCs divide to form the mature sperm. The hub acts as the niche for both GSCs and CySCs and controls their asymmetric division by reciprocal signaling.

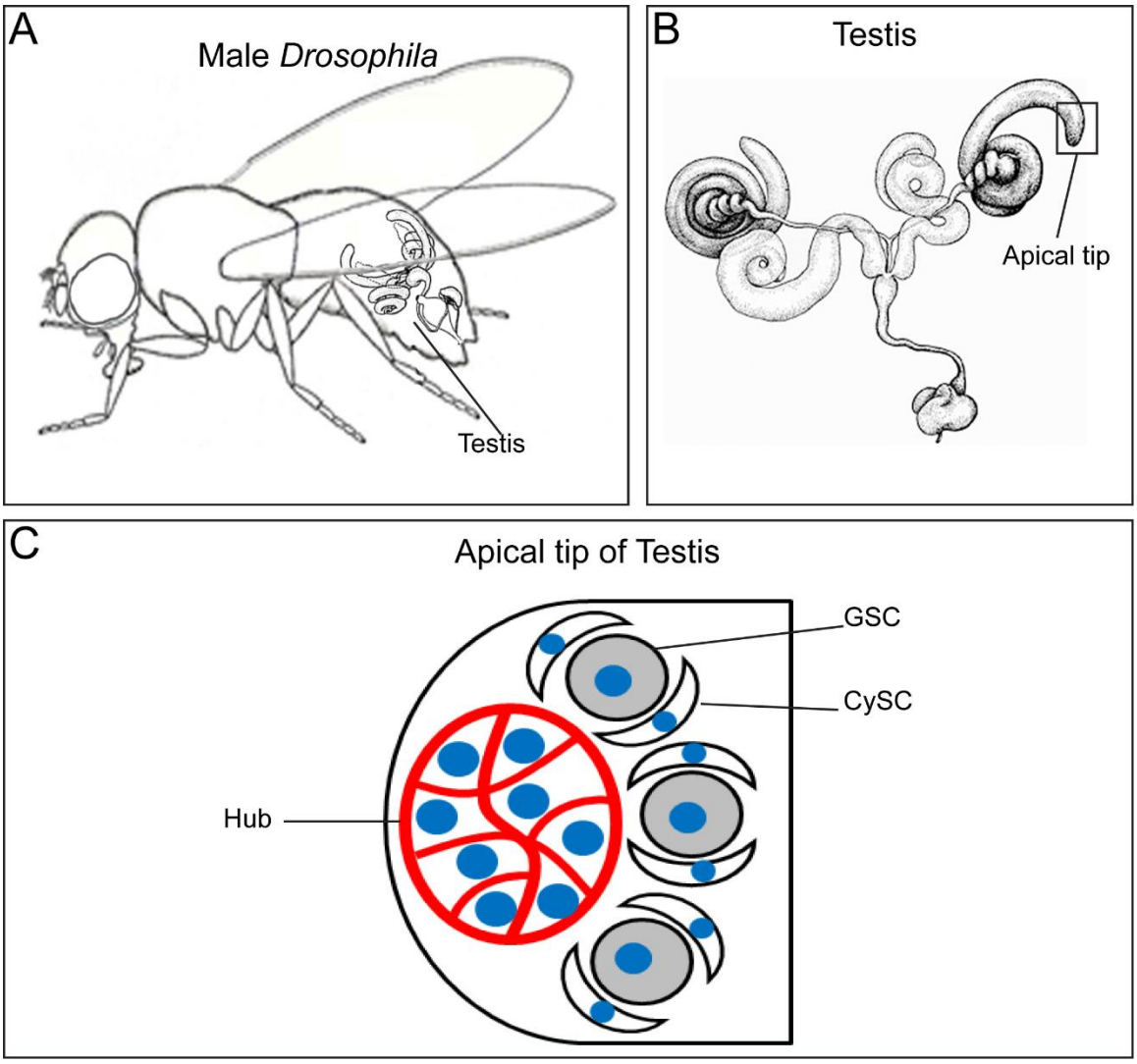
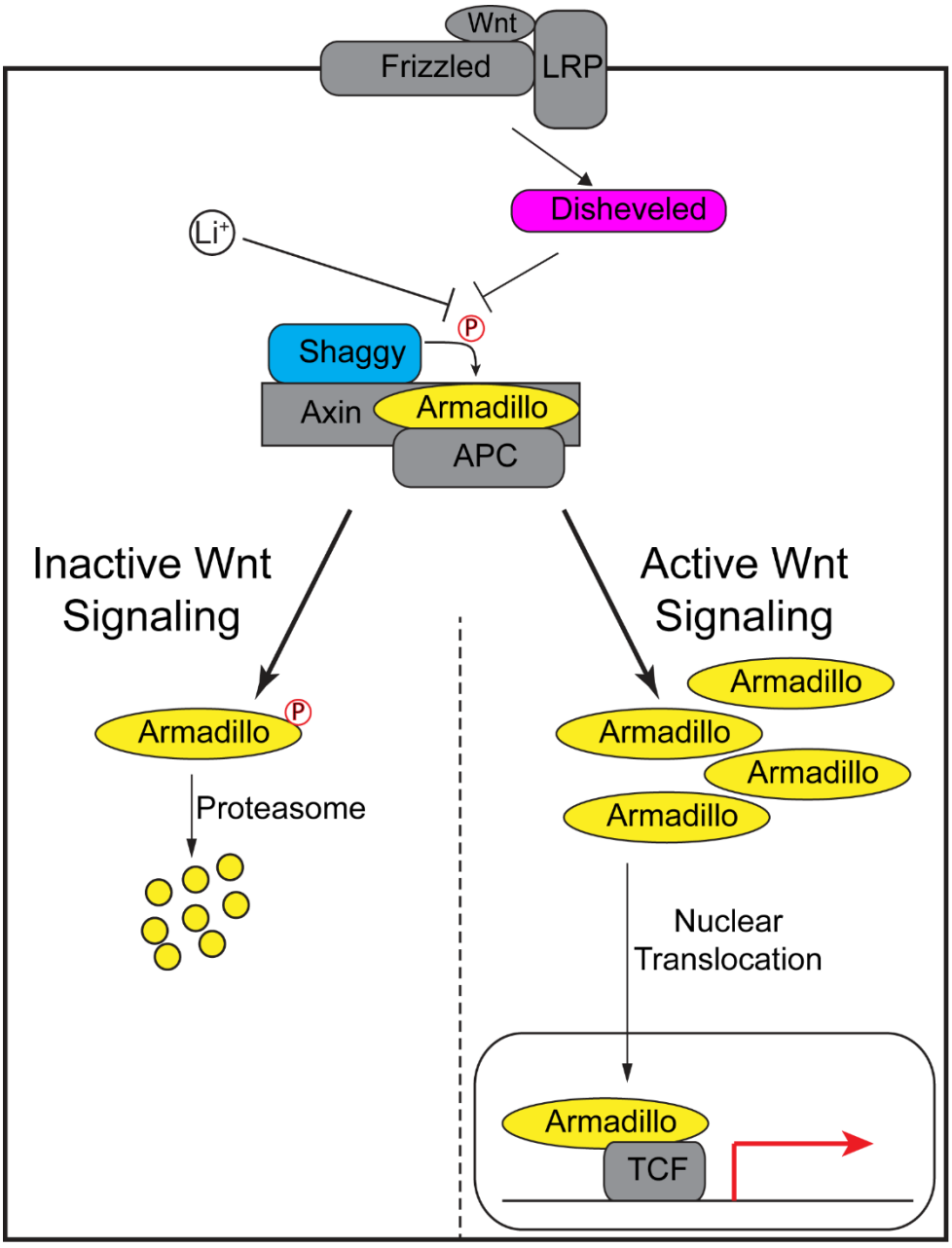


Figure 1.3 *Drosophila* Wnt signaling

Schematic of the *Drosophila* Wnt signaling pathway. Armadillo (Arm, yellow) is the main signal transducer of Wnt signaling. It is bound in a complex with Axin, APC and Shaggy (Sgg, blue) that targets it for proteasome mediated degradation in the absence of Wnt signaling (left side). Wnt signaling is activated when the ligand Wnt binds to the transmembrane receptors Frizzled (Fz) and LRP. This activation leads to Disheveled (Dsh, pink) to be recruited to the membrane and activated. Dsh inhibits Sgg phosphorylation of Arm leading to intracellular accumulation of Arm (right side). Arm then translocates to the nucleus where it associates with a transcription factor TCF and activates transcription of downstream genes (red arrow). Li^+ is an inhibitor of Sgg and inhibits the phosphorylation of Arm and activates Wnt signaling.


Wnt
Frizzled
LRP
Disheveled
Li+
P
Shaggy
Axin
Armadillo
APC
Inactive Wnt Signaling
Active Wnt Signaling
Armadillo
P
Proteasome
Armadillo
Armadillo
Armadillo
Armadillo
Nuclear Translocation
Armadillo
TCF

CHAPTER 2 Materials and Methods

2.1 Fly Husbandry and stocks used

Flies were raised at room temperature and fed a typical molasses, yeast, cornmeal, agar food, with the exception of the following: *D. sechellia* flies were supplemented with reconstituted Noni Fruit (Hawaiian Health Ohana, LLC) (Amlou *et al.* 1998). All fly stocks with their infection statuses and sources are shown in Table 2.1.

2.2 Adult *Drosophila* cultures

2.2.1 Ageing of flies for polar cell tropism studies

F0 parents were mated at 25°C and F1 progeny were raised at 25°C. After eclosion, the F1 were aged at 25°C for seven days. Ovaries were dissected out of these adults on day seven.

2.2.2. Gal4-UAS genetic cross

Unless otherwise noted, virgin F0 females were collected from room temperature stocks and crosses were kept at 25°C. Newly eclosed F1 adults were collected and males were dissected immediately on day one. F1 females were aged for seven days and then dissected to collect their ovaries. Schematic of an experimental cross is shown in Fig. 2.1 and 2.2A.

2.2.3 Gal4-UAS-Gal80 genetic cross

Unless otherwise noted, virgin F0 females were collected from room temperature stocks and crosses were kept at 18°C (permissive temperature). Newly eclosed F1 adults were collected and aged at 29°C (restrictive temperature) for seven days. On the seventh

day, both males and females were dissected for their gonads. Schematic of an experimental cross is shown in Fig. 2.1 and 2.2A.

2.2.4 Drug treatment

Nutri-Fly German Food Formulation (Genesee Scientific), was prepared according the protocol provided by the manufacturer. LiCl was added to a final concentration of 100mM to the food before dispensing it. An equivalent amount of H_2O was added to the control food. One-day old adult flies were collected at room temperature and transferred to either control or drug food (10 females and 3 males per vial). They were aged for seven days with the drug food. Seven-day-old females were dissected for their ovaries.

2.2.5 Microdissection of *Drosophila* tissues

Adult ovaries and testes were dissected in either plastic or glass dissection wells in Grace's media. Tissue was fixed for 30 mins in 4% paraformaldehyde (PFA, EM grade), 0.2% Triton X-100 and Phosphate buffered saline (PBS). Fix was removed with three washed in PBS+0.2% Triton X-100 (PBT). Tissue was stored in PBT at 4°C.

2.3 Immunofluorescence

2.3.1 General immunohistochemistry

Tissue was blocked for at least 1h with PBT+ 0.2% bovine serum albumin (BSA), 5% normal goat serum (NGS) (PBANG) before incubation with primary antibodies (for dilutions, see Table 2.3). Incubation with primary antibody (diluted in PBANG) was conducted for 3h at room temperature, or overnight at 4°C, nutating. Following incubation, the primary antibody was removed and saved for re-use (up to three times, depending on the antibody). The tissue was quickly washed three times with PBT, followed by three 40-

minute washes with PBT, nutating. The tissue was further blocked with PBANG for 30 min. The tissue was then incubated with secondary antibody conjugated to a fluorophore (diluted in PBANG, for dilutions, see Table 2.3) for 2h at room temperature, nutating, in the dark. Following incubation, the secondary antibody was removed and saved for re-use (up to 3 times) and the tissue was quickly washed three times with PBT. The tissue was then washed overnight in PBT at 4ºC, nutating, in the dark. To label nuclei, tissue was counterstained with Hoechst (10 µg/mL in PBT) (Life Technologies) at room temperature, nutating, in the dark. After removal of the Hoechst, the tissue was quickly washed two times with PBT/BSA. Tissue was then mounted in Prolong Gold (Life Technologies). After mounting media had sufficient time to polymerize (usually overnight), the coverslips were sealed with nail polish.

2.3.2 Fluorescent in situ hybridization of dissected ovaries

Tissue was pre-hybridized in 50% Formamide, 5x Saline Sodium Citrate, 250 mg/l Salmon sperm DNA, 0.5x Denhardt's solution, 20mM Tris-HCl, and 0.1% SDS (Hyb) for 1h. Tissue was incubated in 100ng of each *Wolbachia* probe (see table 2.7) diluted in Hyb for 3h at 37ºC. Tissue was then washed twice for 15 minutes at 55°C in a 1x Saline Sodium Citrate wash with 0.1% SDS and 20mM Tris- HCl and then twice for 15 minutes in a 0.5x Saline Sodium Citrate wash with 0.1% SDS and 20 mM Tris-HCl. Nuclei were counterstained with Hoechst (1 µg/ml, Molecular Probes) added to both the wash solutions at a concentration of 10 µg/mL. Tissue was then mounted in Prolong Gold antifade solution (Life Technologies).

2.3.3 Dual in situ hybridization and immunohistochemistry

Protein immunofluorescence and fluorescence in-situ hybridization protocol was adapted from Zimmerman *et al*. (Zimmerman *et al.* 2013)

Tissue was dissected immediately before immunostaining was performed to reduce the chances of mRNA degradation. All steps of immunostaining were conducted without serum, as the RNases and DNases could degrade the mRNAs. Also, all *in-situ* reagents and buffers were diethylpyrocarbonate (DEPC) treated to eliminate RNases which would otherwise degrade the target RNAs. Tissue was fixed for 20 min in 4% PFA in PBT. Fix was removed, and tissue was washed three times with PBT. Tissue was incubated in primary antibody (diluted in PBT) for 2.5h at room temperature, nutating. Primary antibody was removed, and the tissue was quickly washed three times with PBT and then three 30-minute washes were performed with PBT, nutating. Tissue was then incubated for 1.5h in secondary antibody (diluted in PBT), nutating, in the dark. Secondary antibody was removed, and three quick washes were performed with PBT. Then three 20-minute washes were performed with PBT. The tissue was then fixed for 30 min with 4% PFA in PBT. In situ hybridization was further performed on this tissue as per Section 2.3.2

2.4 Quantification of *Wolbachia* density

2.4.1 Imaging equipment

All images were acquired using an Olympus Fluoview FV1000 confocal microscope. Imaging parameters once established were kept consistent for all images of the entire experiment.

2.4.2 Density analysis in the hub

Z stacks of representative images were analyzed for *Wolbachia* density using MatLab software, as defined by Frydman, *et al*. 2006. Hubs were distinguished by a hub marker. Manual masks were drawn for the hub and the surrounding region (see Fig. 2.5). Relative *Wolbachia* density in the hub was calculated as per the given formula

$$Relative\ Wolbachia\ density\ in\ the\ hubs = \frac{Voxel\ density\ of\ Wolbachia\ in\ hubs}{Voxel\ density\ of\ Wolbachia\ in\ surrounding\ cells}$$

2.4.3 Density analysis in the ovarian polar cells

All images were acquired on a FV1000 confocal microscope (Olympus) using the 60X objective (NA 1.42). Image acquisition parameters were kept constant for all images within each *Wolbachia* strain.

For representative images: 4-8 Z stacks 1µm apart encompassing the PC were combined and images were processed in Photoshop CS6 to eliminate neighboring egg chambers not relevant to the image being shown. This was done for clarity.

Wolbachia density analysis: For every image, 4-8 Z stacks of 1µm each encompassing the polar cells were collected. Manual thresholding was performed to subtract background nonspecific staining and to ensure that only pixels with *Wolbachia* staining were accounted for. Manual masks were drawn over the polar cells and the follicle cells using the polar cell marker (FasIII) and DNA (see Fig. 2.6). *Wolbachia* density was measured in each mask using MatLab and relative density was calculated based on the equation below:

$$Relative\ Wolbachia\ density\ in\ the\ polar\ cells = \frac{Voxel\ density\ of\ Wolbachia\ in\ Polar\ cells}{Voxel\ density\ of\ Wolbachia\ in\ Follicle\ cells}$$

2.5 *Wolbachia* quantification by quantitative PCR

DNA was extracted from ovaries or whole flies using the Qiagen DNeasy Blood & Tissue kit. *Wolbachia* levels were quantified by levels of *Wolbachia* gene *wsp*. All qPCR reactions were performed on the ABI 7900HT system. PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) was used according to manufacturer specifications. 5ng DNA input was used with cycle conditions: denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing and extension at 60°C for 1 minutes. Reactions were run in triplicates of three independent experiments. Housekeeping gene 14-3-3 was used for *D. melanogaster* for normalization purposes. DNA sequences of all primers used are in Table 2.5.

2.6 Quantitative RT-PCR

RNA was extracted from ovaries or whole flies using the Qiagen RNeasy kit. All qRTPCR reactions were performed on the ABI 7900HT system. PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and was used according to manufacturer specifications. SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific) was added in the reaction mix for a One-Step qRTPCR according to manufacturer specifications. 5ng RNA input was used with cycle conditions: DNA synthesis step at 50ºC for 5mins, denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing and extension at 60°C for 1 minutes. Reactions were run in triplicates of three independent experiments. DNA sequences of primers used are in Table 2.6.

2.6 Cell culture

Drosophila cell lines (JW18) were grown in Shields and Sang M3 insect media (Sigma S3652) at 28ºC. For drug treatment, the cells were plated in 96-well plates. 0 or 10mM LiCl was supplemented in the growth medium and cells were grown for 1 week before harvesting and extracting DNA. *Aedes aegypti*i cell lines (Aag2) infected with wAlbB were grown in Schneider's Insect Media (Sigma S9895) with 0mM or 10mM LiCl supplementation. DNA was extracted from cells harvested after 5 days. *Aedes albopictus* cell lines (Aa23) infected with wAlbB were also grown in Schneider's Insect Media (Sigma S9895) with 0mM or 10mM LiCl supplementation. DNA was extracted from cells harvested after 2 days.

2.7 Western blotting

Fly samples were homogenized and incubated for 20 minutes in ELB buffer (150mM NaCl, 50mM Hepes pH7, 5mM EDTA, 0,1% NP-40) containing protease inhibitor, 1mM PMSF and 1mM DTT. Supernatant containing proteins was collected after centrifugation at 12000g for 10min. Protein extracts, separated by SDS-PAGE and transferred onto nitrocellulose membrane according to manufacturer's protocol (Bio-Rad), were blocked with condensed milk powder in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) overnight at 4°C and then probed with primary antibodies diluted in TBST (TBS with 0.5% Tween 20) for an hour (dilutions in Table 2.4). After removal of antibodies, the membrane was washed three times with TBST and incubated in HRP conjugated secondary antibody for 30 minutes at room temperature. Finally, membrane was washed in TBST for three times and developed with Western Lightening Plus-ECL (PerkinElmer) according to the

manufacturer's protocol. The blots were then exposed to photographic film and developed. The films were scanned, and quantification performed using ImageJ.

2.8 Proteasome activity assay

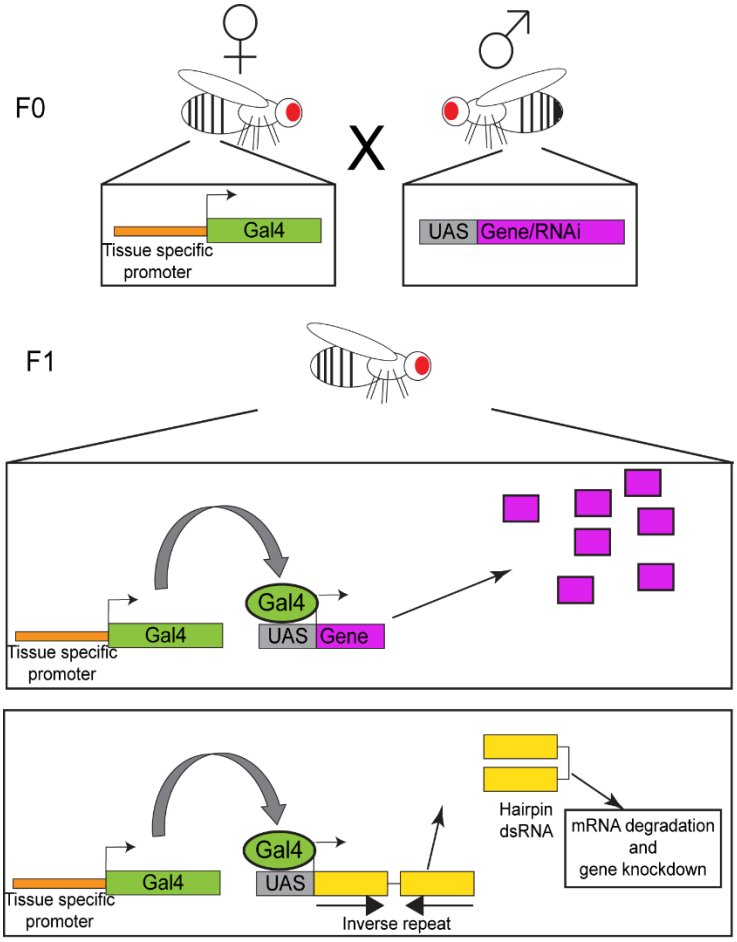
Ovaries from 15 seven-day old females were dissected. The ovaries were crushed in PBS+0.1% TritonX100, centrifuged at 12000g for 5 min, and supernatant collected. After protein estimation with Bradford assay, approximately 20µg of protein was added to 200µl of reaction buffer (50mM Tris pH7.5; 5mM $MgCl_2$; 1mM DTT; 100µM Z-LLE-AMC). The reaction was set up in 96-well Corning® black fluorescence plates. Proteasome activity was measured by quantifying fluorescence of free AMC in a SpectraMax M5 plate reader (Ex:380nm; Em:460nm). Each replicate was repeated with a proteasome inhibitor, MG132. Background fluorescence reading with MG132 was subtracted from each replicate.

2.8 Statistical analyses

For all P-values listed, the statistical test used is indicated. Statistical tests were performed using Microsoft Excel.

Figure 2.1 Gal4-UAS system

General scheme for the Gal4-UAS cross and system. A tissue specific promoter drives the expression of a Gal4 transcription factor in one parent. The other parent has an upstream activating sequence (UAS) followed by a gene or RNAi of interest. In the F1 progeny, the Gal4 protein expressed in tissues of interest binds to the UAS leading to the expression of the gene of interest (purple). In case of RNAi, the UAS is upstream of an inverse repeat complementary to the mRNA to be targeted. This forms a hairpin dsRNA that enters the endogenous RNAi pathway leading to knockdown of the gene. The various tissues targeted in this thesis and their corresponding Gal4 drivers are indicated in orange.



Hub: upd-Gal4
Polar cells: upd-Gal4
Germline: nos-Gal4
Germline stem cell niche: bab-Gal4

Figure 2.2 Generic Gal4-UAS cross

(A) Cross scheme for a RNAi construct on the 3rd chromosome. (B) Cross scheme for a RNAi construct on the 3rd chromosome with a Gal80ts construct on the 2nd. This allows for temporal control of RNAi expression. Similar schemes would be used if the construct was on the 2nd chromosome. Gal80ts is a temperature sensitive repressor of Gal4. At 18ºC, Gal80ts represses gene expression. Whereas at 29ºC, it is inactivated, and gene expression is resumed. upd: unpaired driver; tub: tubulin driver; MKRS: a third chromosome balancer; →: Y chromosome.

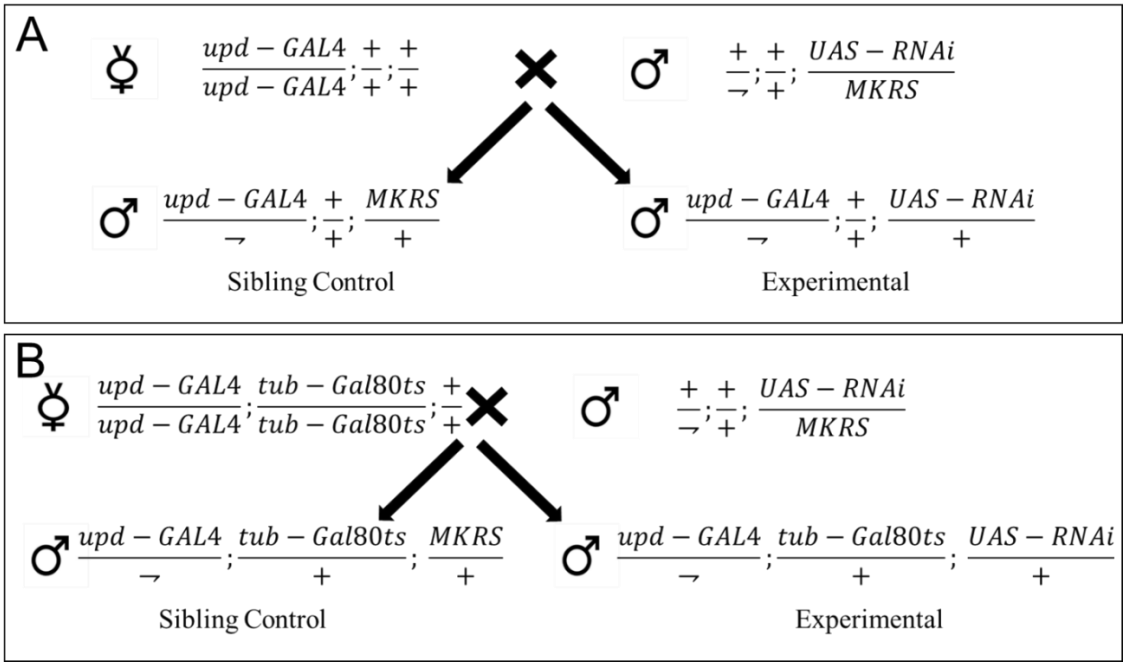


Figure 2.3 Diagram of experimental setup

(A) Schematic of an experimental setup for a Gal4-UAS cross. 10 virgin females and 3 males were mated in a vial for 3 days following which the F0 parents were removed. Newly eclosed (NE) F1 progeny were collected and moved to a new vial. For hub studies, one to two-day old males were dissected. For PC and germline studies, seven-day old females were dissected. (B) Schematic of an experimental setup for a Gal4-UAS-Gal80^{ts} cross. 10 virgin females and 3 males were mated for 3 days at 18ºC, the permissive temperature of Gal80^{ts}. F1 NE adult were collected and moved to a new vial which was kept at 29ºC, the Gal80^{ts} restrictive temperature. Seven-day old adults were dissected for hub, PC, and germline studies.

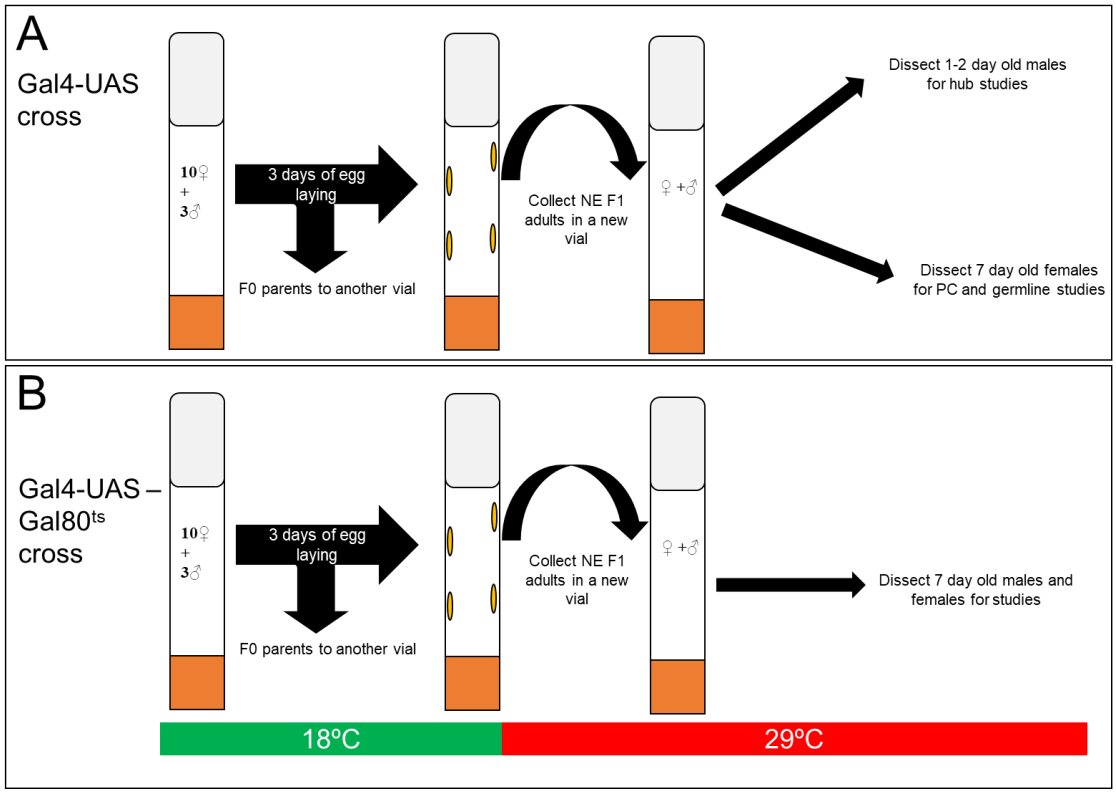


Figure 2.4 *Wolbachia* FISH control

(A-C) Staining of *Wolbachia* infected egg chambers with the *Wolbachia* 16S rRNA FISH probes. (D) Staining of a *Wolbachia* uninfected ovary shows that there is no nonspecific binding of the *Wolbachia* 16S rRNA probe (green channel). Polar cells are labeled by FasIII in red and DNA in blue. Scale bars = 10μm

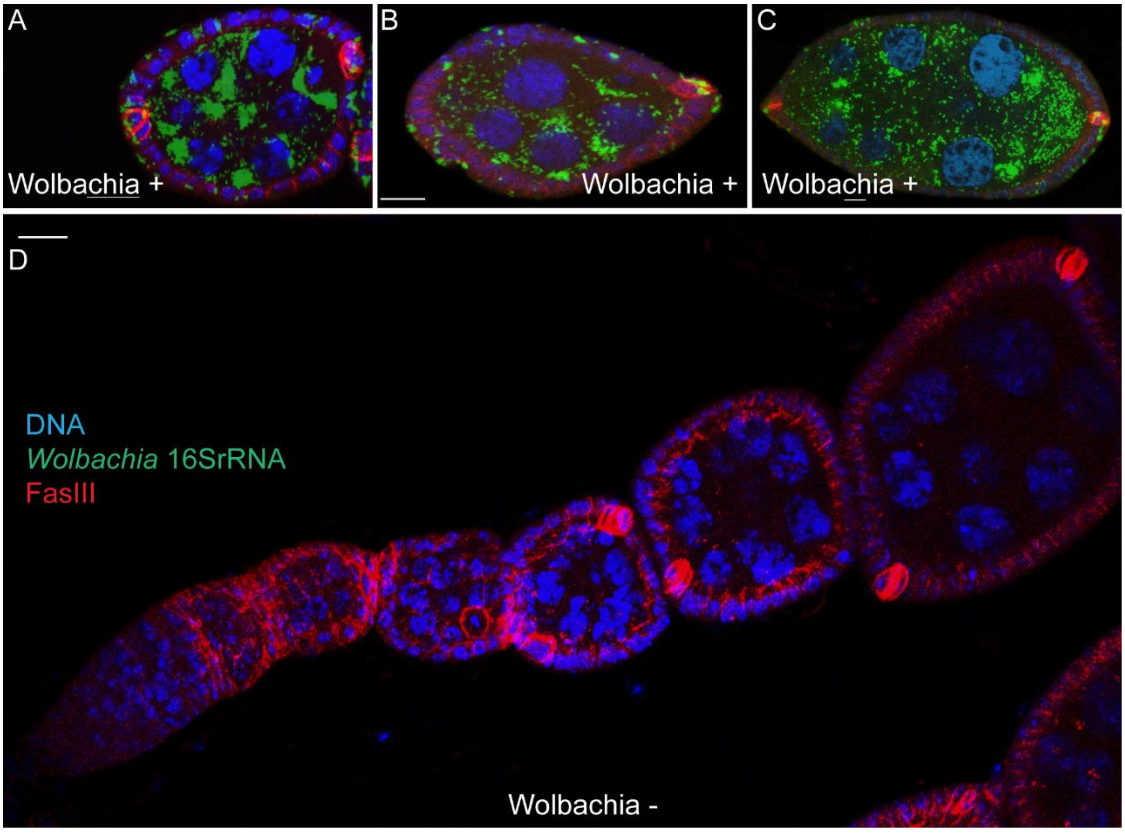
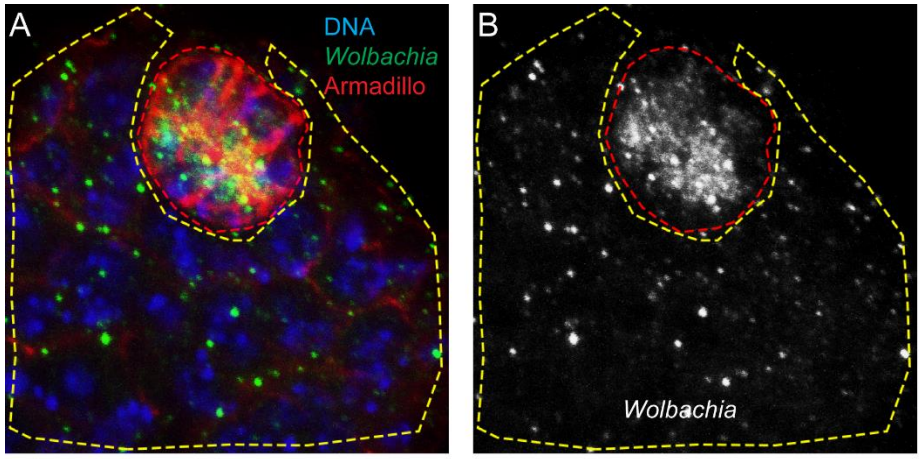


Figure 2.5 Quantification of *Wolbachia* density in the hubs

(A) A confocal image of the hub stained for *Wolbachia* (anti-Hsp60, green) and a hub marker (anti-armadillo, red). Manual masks drawn over Hub (red dotted line) and the surrounding area (yellow dotted line). (B) *Wolbachia* channel alone. Relative *Wolbachia* density in the hub was calculated using the formula given.

Voxel density is the sum of *Wolbachia* signal intensity of all the pixels delimited by the drawn masks at all focal planes spanning the hub (3-5 Z sections 1μm apart). Image analysis, mask drawing, and quantifications were performed using MatLab.

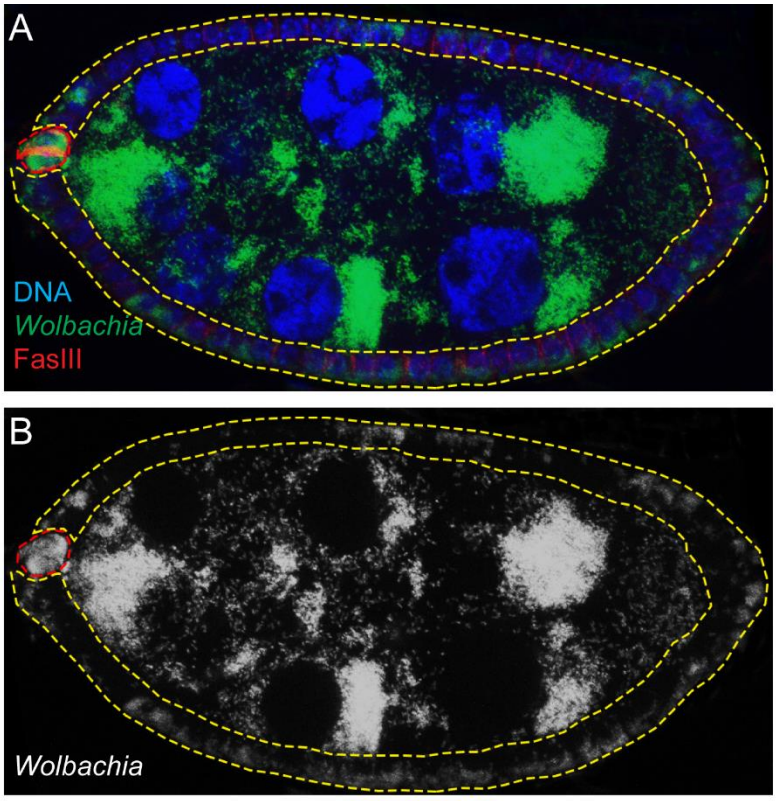


$$\text{Relative } \textit{Wolbachia} \text{ hub density} = \frac{\text{Voxel density of } \textit{Wolbachia} \text{ in hub (Red dotted line)}}{\text{Voxel density of } \textit{Wolbachia} \text{ in surrounding (Yellow dotted line)}}$$

Figure 2.6 Quantification of *Wolbachia* density in the polar cells

(A) A stage 8 egg chamber with manual masks drawn over polar cells (PCs, red dotted line) and lateral follicle cells (FC, yellow dashed line). The posterior PCs are not visible here as they lie in Z-stacks not displayed here. (B) Image of *Wolbachia* channel only. Relative *Wolbachia* density in the PCs was calculated using the equation shown.

Voxel density is the sum of *Wolbachia* signal intensity of all the pixels delimited by the drawn masks at all focal planes spanning the PCs (4-8 Z sections 1μm apart). Image analysis, mask drawing, and quantifications were performed using MatLab.



$$\text{Relative } \textit{Wolbachia} \text{ PC density} = \frac{\text{Voxel density of } \textit{Wolbachia} \text{ in PC (Red dotted line)}}{\text{Voxel density of } \textit{Wolbachia} \text{ in lateral FC (Yellow dotted line)}}$$

Table 2.1 *Drosophila* species used for analysis

***Drosophila* species**	***Wolbachia* strain**	**Source**	**Stock center reference#**
D. melanogaster	*w*Mel	Frydman Lab	-
D. melanogaster	*w*MelCS	Sullivan Lab	-
D. melanogaster	*w*MelPOP	Luis Teixeira	-
D. simulans	*w*No	San Diego Stock Center	14021-0251.198
D. simulans	*w*Ri	San Diego Stock Center	14021-0251.169
D. sechellia	*w*Sh	San Diego Stock Center	14021-0248.08
D. mauritiana	*w*Mau	San Diego Stock Center	14021-0241.01
D. teissieri	*w*Tei	San Diego Stock Center	14021-0257.00
D. tropicalis	*w*Wil	San Diego Stock Center	14030-0801.01
D. yakuba	*w*Yak	Virginie Orgogozo	-

Table 2.2 Transgenic flies used in this analysis

Shorthand name	**Genotype**	**Source**	**Frydman lab stock number**
upd-Gal4 Driver	$\frac{upd-Gal4}{upd-Gal4};;$	Dr. Erika Matunis John Hopkins School of Medicine, Baltimore, MD	391 wMel 392 wMelCS
upd-Gal4 Driver with tub-Gal80^{ts}	$\frac{upd-Gal4}{upd-Gal4};\frac{tub-Gal80ts}{tub-Gal80ts};$		430 wMel 431 wMelCS
nanos-Gal4 Driver	$;\frac{NGT40}{NGT40};\frac{nos-Gal4}{nos-Gal4}$	Dr. Kim McCall Boston University	418 wMel 419 wMelCS
bab-Gal4 Driver	$;;\frac{bab-Gal4}{TM3,Sb}$	BDSC #6802	276
*arm*RNAi on III	$;;\frac{pTRiP[JFO1251]attp2}{TM3,Sb}$	BDSC #31304	363
Arm^{S10} on II	$;\frac{UAS-ArmS10}{UAS-ArmS10}$	Dr. Norbert Perrimon Harvard Medical School	383
*dsh*RNAi on II	$y[1],v[1];;\frac{\text{P\{TRiP. JF01253\}attP2}}{\text{P\{TRiP. JF01253\}attP2}}$	BDSC #31306	507
*sgg*RNAi on III	$y[1],v[1];;\frac{P\{TRiP.JF01256\}attP2}{TM3,Ser}$	BDSC#31309	510
STAT-GFP on II	$;;\frac{P\{w[+mC]=10XStat92E-GFP\}1}{P\{w[+mC]=10XStat92E-GFP\}1}$	BDSC#26197	379

Table 2.3 Antibodies used for immunohistochemistry

Name	Dilution	Host	Source
Primary Antibody			
Hsp60	1:100	Mouse	Sigma cat#H3524
E-Cadherin	1:100	Rabbit	Santa Cruz cat#sc-7870
Armadillo	1:100	Rabbit	Santa Cruz cat#sc-28653
N-Cadherin	1:200	Rat	DSHB cat#DN-EX#8
Lamin C	1:100	Mouse	DSHB cat#LC28.26
FasIII	1:2000	Mouse	DSHB cat#7G10
Secondary Antibody			
Anti-mouse, Alexa 488	1:500	Goat	ThermoFisher Cat#A-32723
Anti-rabbit, Alexa 568	1:500	Goat	ThermoFisher Cat#A-11011
Anti-rat, Alexa 568	1:500	Goat	ThermoFisher Cal#A-11077
Anti-rat, Alexa 647	1:500	Goat	ThermoFisher Cal#A-21247
Anti-rabbit, Alexa 647	1:500	Goat	ThermoFisher Cat#A-21244

Table 2.4 Antibodies used for Western Blots

Name	Dilution	Host	Source
Primary Antibody			
pGSK3b(ser21/9)	1:1000	Rabbit	Cell Signaling Technologies #9331
GSK3 clone 4G-1E	1:1000	Mouse	Millipore #05-412
Secondary Antibody			
Anti-rabbit HRP	1:10000	Goat	Fisher Cat#50-904-9302
Anti-mouse HRP	1:10000	Goat	Fisher Cat#50-904-9305

Table 2.5 Primers used for qPCR

Name	Species	Sequence
Wsp_F	*Wolbachia w*Mel	TTGGAACCCGCTGTGAATGA
Wsp_R	*Wolbachia w*Mel	CCGAAATAACGAGCTCCAGCA
wAlb_F	*Wolbachia w*AlbB	GGTTTTGCTGGTCAAGTAA
wAlb_R	*Wolbachia w*AlbB	GCTGTAAAGAACGTTGATC
wStr_F	*Wolbachia w*Str	TCAAGCAAAAGCTGGTGTTAGC
wStr_R	*Wolbachia w*Str	CAGCATCATCCTTAGCTGCC
14-3-3_F	*Drosophila melanogaster*	CATGAACGATCTGCCACCAAC
14-3-3_R	*Drosophila melanogaster*	CTCTTCGCTCAGTGTATCCAAC
Rps6_F	*Aedes aegypti*	AGTTGAACGTATCGTTTCCCGCTAC
Rps6_R	*Aedes aegypti*	GAAGTGACGCAGCTTGTGGTCGTCC

Table 2.6 Primers used for qRT-PCR

Name	Gene	Sequence
Upd_F	Unpaired	ACTTCGACGAGAACACCACC
Upd_R	Unpaired	TGATTGAAGCTCTGCCTGG
Imp_F	IMP	GGTGGGCCGTATCATTGG
Imp_R	IMP	TCACGCGCTGCAATTCC
Rpl32_F	Ribosomal protein 32	ATGCTAAGCTGTCGCACAAATG
Rpl32_R	Ribosomal protein 32	GTTCGATCCGTAACCGATGT

Table 2.7 In situ hybridization oligonucleotides

Name	Species	Sequence
Wpan16S887	*Wolbachia*	5'-ATCTTGCGACCGTAGTCC-3'
Wpan16S450	*Wolbachia*	5'-CTTCTGTGAGTACCGTCATTATC -3'

Probes are labeled on the 5' end with Cy3 or Cy5 and were purchased from Integrated DNA Technologies. Probes were designed against conserved regions of the 16S gene and work for a wide variety of *Wolbachia* strains. Probe sequences were adapted from (Moreira *et al.* 2009).

CHAPTER 3

Polar cell fate triggers *Wolbachia* intracellular accumulation

Portions of this chapter were previously published in (Kamath *et al.* 2018)

3.1 Introduction

During their life cycle, all animals host a variety of microorganisms in their bodies. These microbes preferentially localize to specific tissues or organs, a phenomenon termed tissue tropism. Understanding the mechanisms and consequences of tissue tropism is key to elucidating host-microbe interactions. One of the largest pandemics on the planet is a maternally transmitted alphaproteobacteria belonging to the genus *Wolbachia* which infect a large fraction of invertebrates, including parasitic filarial worms, and insect vectors of infectious diseases (Moreira *et al.* 2009, Kambris *et al.* 2010, Hughes *et al.* 2011, Walker *et al.* 2011). *Wolbachia* are stably maintained in host populations and have a profound effect on host biology, including their evolution, physiology, reproduction, immunity, and development (Werren *et al.* 2008). During evolution, *Wolbachia* have developed tropism to specific host tissues to facilitate their efficient vertical transmission (Hadfield and Axton 1999, Veneti *et al.* 2004, Ferree *et al.* 2005, Frydman *et al.* 2006, Serbus and Sullivan 2007, Werren *et al.* 2008). Germline infection in the gonads is essential for maternal transmission. However, *Wolbachia* also infect several somatic tissues of the host (reviewed by Dobson *et al.* 1999, Cheng *et al.* 2000, Clark *et al.* 2005, Espino *et al.* 2009, Hosokawa *et al.* 2010, Fischer *et al.* 2011, Pietri *et al.* 2016).

In the *Drosophila* gonads, *Wolbachia* infect the stem cell niches, microenvironments that support the stem cells, at high levels (Frydman *et al.* 2006, Fast *et*

al. 2011, Toomey *et al.* 2013, Toomey and Frydman 2014). In females, these encompass both the niche supporting the somatic and germline stem cells (Fig. 3.1A-C), while in the male there is a single niche for both somatic and germline stem cells, known as the hub (Fig. 3.1D,E). The somatic stem cell niche (SSCN) harbors the somatic stem cells (SSC) which generate all the somatic cells that envelope the germline and secrete the egg shell (Fig. 3.1A). *Wolbachia* tropism to the SSCN has been shown to be important in their transmission to the germline and therefore to the next generation (Toomey *et al.* 2013). Moreover, previous work has demonstrated that upon recent infection, *Wolbachia* first colonize the somatic stem cell niche (SSCN) of adult *Drosophila melanogaster* (Frydman *et al.* 2006).

These observations were seen in already differentiated niches in adults. The kinetics of *Wolbachia* tropism to the niches during their specification and development has not been defined. This analysis is not easily accomplished because the morphogenesis of these niches occurs prior to adulthood. The SSCN is specified during pupal development in the presence of differentiated germ cells (Nystul and Spradling 2007, Sahai-Hernandez and Nystul 2013, Vlachos *et al.* 2015). Furthermore, the SSCN precursor cells are not predefined, making it difficult to study tropism during niche morphogenesis (Sahai-Hernandez and Nystul 2013, Vlachos *et al.* 2015). The male stem cell niche, hub, is also infected with *Wolbachia* at high densities, however the specification of the hub occurs in mid-embryogenesis (Le Bras and Van Doren 2006, Sheng *et al.* 2009), and its development spans multiple life stages of the insect. Therefore, to determine the kinetics of *Wolbachia* accumulation to these somatic tissues during development requires quantification of

multiple developmental stages, including pupal stages, making stem cell niches a challenging system to study *Wolbachia* tropism during their development.

We probed for *Wolbachia* tropism to other cell types during *Drosophila* oogenesis, where most developmental stages of different cell types from stem cell division to egg maturation can be observed in a single individual. Each ovary has about 14-16 ovarioles which develop in parallel and one can follow all the morphogenetic events of various cell types in a single insect (Spradling 1993, Wu *et al.* 2008). Furthermore, it is a well characterized system with a vast array of cellular and molecular tools and markers available for each cell type. Each *Drosophila* egg begins as a 16-cell germline cyst, from which one cell will become the oocyte and the remainder will become the supporting nurse cells. The cyst then gets encapsulated by a monolayer of somatic follicle cell precursors (blue cells in Fig. 3.1A). As the cyst exits the germarium, a population of follicle cells on either pole ceases to proliferate, differentiating into a pair of polar cells (PC). From the same precursor population, cells differentiate into a stalk between the consecutive chambers. The other follicle cells, known as lateral follicle cells, remain undifferentiated and keep dividing to encapsulate the germline (Margolis and Spradling 1995, Gonzalez-Reyes and St Johnston 1998, Grammont and Irvine 2002, Xi *et al.* 2003). In each ovariole, we can observe most stages of development of various cell types including the PCs from its origin to its maturation. Here we describe preferential *Wolbachia* targeting in the PCs. This makes the PCs a powerful system to study *Wolbachia* tropism during development. Moreover, there are genetic tools that allow easy manipulation of these cells, including the capability to induce ectopic PCs.

Wolbachia PC tropism is a novel system to study host-*Wolbachia* interactions in the somatic cell types. Using confocal microscopy and transgenic flies to generate ectopic PCs, we demonstrate that *Wolbachia* show remarkable specificity and coordination of their accumulation in PCs with specific developmental events during oogenesis. Furthermore, we show that *Wolbachia* PC tropism is evolutionarily conserved in the *Drosophila* genus, but is absent in another dipteran, the mosquito *Culex pipiens*.

3.2 *Wolbachia* tropism to the PCs is pervasive across the *Drosophila* genus in most species tested.

In the fly ovary, usually *Wolbachia* accumulate at high levels in the germline. Upon imaging *Wolbachia w*MelPop strain in *Drosophila melanogaster* we noticed a consistent high *Wolbachia* accumulation at the polar regions of the follicular epithelium of the egg chamber, reaching levels equivalent to germline infection (Fig. 3.2B). The pattern of this accumulation was consistent with the localization of the polar cells (PCs) (Schematic in Fig. 3.2A). Using an antibody against Fasciclin III (FasIII) to label PCs (Patel *et al.* 1987, Ruohola *et al.* 1991), we confirmed that *Wolbachia*, although present at low levels in the lateral follicle cells, accumulate at high levels in the PCs (Fig. 3.2B).

To address if this tropism is evolutionarily conserved, we surveyed 10 *Wolbachia* strains that naturally infect seven *Drosophila* species for their PC tropism. Using FISH to label *Wolbachia* and FasIII staining to label polar cells (see material and methods), we quantitatively assessed *Wolbachia*'s PC tropism in the ovaries of all 10 *Wolbachia*–*Drosophila* pairs. In every ovary analyzed, we found the presence of *Wolbachia* in PCs (Fig. 3.2B-K). To quantify *Wolbachia* levels, voxel density from representative Z stacks

were determined using an image analysis software (see Materials and Methods). We used stage 8 as a representative stage of oogenesis. By comparing relative *Wolbachia* levels in the PC to lateral follicle cells, we found that *Wolbachia* were enriched in the PCs relative to the follicular epithelium in each of the *Drosophila-Wolbachia* pairs except for *Dsim* wRi (Fig. 3.2L). Fitting the PC tropism phenotype to the *Wolbachia* phylogenetic tree (Fig. 3.3) (Paraskevopoulos *et al.* 2006) indicated that the ancestral strain of *Wolbachia w*Ri had PC tropism and this feature was lost in *w*Ri, most likely during the separation between *w*Ri and its closest living relative analyzed, *w*Sh (Fig. 3.3). These data indicate a strong selective pressure for an evolutionary conserved *Wolbachia* tropism to PCs.

Polar cells (PCs) are believed to be a unique feature of the *Diptera* order. Although PCs have been identified in all dipterans investigated so far they have never been found in non-dipteran insects (Jaglarz *et al.* 2008). Here we identified PC tropism in most *Drosophila* species, a higher dipteran (*Brachycera*). To determine if *Wolbachia* PC tropism is pervasive outside the *Drosophila* genus, we investigated the mosquito *Culex pipiens*, a lower dipteran (*Nematocera*), an evolutionarily distant species. *C. pipiens* are infected with *w*Pip, a *Wolbachia* strain highly divergent from *w*Mel. Putative PCs have been visualized in the mosquito *Culex pipiens* by transmission electron microscopy (Soumare and Ndiaye 2005). We found that antibody against *Drosophila* N-cadherin labels two follicular cells at opposing poles of *Culex* follicles, possibly the mosquito PCs. Double staining of *Wolbachia* and N-cadherin shows that *Wolbachia* do not accumulate in the putative PCs of *Culex pipiens* (Fig. 3.4). Therefore, PC tropism is either an evolutionary novelty that occurred in higher dipterans or it was lost in *Culex.*

3.3 *Wolbachia* tropism to the PCs occurs very early in development.

To characterize this novel *Wolbachia* tropism, we carried out further analyses in *Drosophila melanogaster* and its 3 *Wolbachia* endosymbiont strains, *w*Mel, *w*MelCS and *w*MelPop. The development of the egg chamber can be divided into 14 stages (Fig. 3.5A) (King 1970, reviewed by Spradling 1993). PCs are differentiated early in follicular epithelium morphogenesis (Margolis and Spradling 1995, Grammont and Irvine 2002). Two pairs of PCs can be unequivocally distinguished from the remaining follicular epithelium by stage 4 of oogenesis by immunostaining for a membrane marker FasIII (Ruohola *et al.* 1991, Besse and Pret 2003, Khammari *et al.* 2011). We quantified *Wolbachia* density in the PCs relative to the lateral follicle cells at various stages of oogenesis. In all three strains of *Wolbachia* tested (*w*Mel, *w*MelCS and *w*MelPop), we observed an elevated *Wolbachia* density in the PCs relative to the lateral follicle cells starting from Stage 4 up until Stage 10 of oogenesis (Fig. 3.5B-F). This observation shows that *Wolbachia* infect PCs very early in development and maintain a high titer throughout their development.

3.3 *Wolbachia* tropism to the PCs is coordinated with specific developmental events of mid oogenesis

To further study *Wolbachia* accumulation kinetics in PCs, we quantified *Wolbachia* titers in PCs relative to lateral follicle cells in the same egg chamber at various stages of oogenesis (4,5,8,9 and 10). Normalization to lateral follicle cells was performed to account for variability of staining and confocal image acquisition across different experiments (see material and methods). In all three strains of *Wolbachia*, we found that bacterial density in

the PCs relative to lateral follicle cells increased steadily from stage 4 to stage 10 (Fig. 3.5G-I). This shows that *Wolbachia* have a preferential tropism to the PCs and increase in density as they progress through development.

As the egg chamber progresses through development, the lateral follicle cells increase about 20-fold between stages 4 and 6 (Xi *et al.* 2003, Assa-Kunik *et al.* 2007, Wu *et al.* 2008). During stages 9 and 10, the other lateral follicle cells become more columnar, undergo endoreplication and increase in size (Gonzalez-Reyes and St Johnston 1998, Wu *et al.* 2008). Between stage 4 and 10, the number of PCs, however, remain at four (two at each pole). The increased number and size of lateral follicle cells could inflate the relative *Wolbachia* density in the PCs. To address this, we compared the *Wolbachia* densities in lateral follicle cells over different stages of oogenesis. For *w*Mel and *w*MelCS, we observed no significant decrease in *Wolbachia* density in these over the different stages of development (Fig. 3.6A,B). However, in the *w*MelPop strain, we observed that there was a steady decrease of *Wolbachia* density in lateral follicle cells throughout development (Fig. 3.6C). *w*MelPop is a pathogenic strain of *Wolbachia* which replicates at an extremely high rate and leads to premature deaths of infected individuals (Woolfit *et al.* 2013). In the PC, it reaches a high density early in oogenesis and maintains these levels throughout oogenesis, even as the levels in the surrounding lateral follicle cells decreases. Together these results show that our measurements of *Wolbachia* tropism to the PCs is not augmented by the normalization to lateral follicle cells.

Interestingly, we observed a particularly large increase in *Wolbachia* density between stages 8 and 9 in the PCs (Fig. 3.5G-I). The *Wolbachia* density increased by about three-fold in *w*Mel and wMelCS and two-fold in wMelPop. In stage 9, the anterior PCs along with a few surrounding cells (called border cells) migrate amidst the nurse cells to ultimately associate with the anterior of the oocyte (Fig. 3.8). During this migration, they are very closely associated with the germline. (Montell *et al.* 1992, Montell 2003). The border cell migration event would allow for a perfect opportunity for *Wolbachia* to traverse from PCs to the germline. Using previously described kinetics of oogenesis (Lin and Spradling 1993), we plotted *Wolbachia* density as a function of time from stage 4 to 10 (Fig. 3.9). This analysis shows that *Wolbachia* PC density increases moderately (*w*Mel, 1.12-fold; *w*MelCS, 1.68-fold; *w*MelPop, 1.04-fold) between stages 5 and 8 over a period of ~17 hours (Fig. 3.9). However, between stages 8 and 9, *Wolbachia* PC density increases rapidly (*w*Mel, 2.73-fold; *w*MelCS, 2.32-fold; *w*MelPop, 2.18-fold) in ~8 hours. Remarkably, this shows that *Wolbachia* coordinate their replication and accumulation with specific host developmental events.

3.4 *Wolbachia* reside at equal density in anterior and posterior PCs

In each egg chamber, there are two pairs of PCs at each pole. The anterior PCs are required for the migration of four to eight adjacent cells termed the border cells (reviewed by Montell 2003) during stage 9. The posterior PCs help localize the oocyte, and then participate in a reciprocal signaling with the oocyte to re-organize the oocyte cytoskeleton thereby establishing the anteroposterior and dorsoventral axes of the oocyte (Gonzalez-Reyes *et al.* 1995, Roth *et al.* 1995). As each set of PCs have distinct functions, we

hypothesized that the *Wolbachia* levels would differ between the anterior and posterior PCs. By comparison we were unable to identify differences in *Wolbachia* density between the anterior and posterior PCs (Fig. 3.10A-F). Upon quantification, we observed that, in most stages of oogenesis, there was no significant difference in *Wolbachia* density between the anterior and posterior PCs (Fig. 3.10G-I). Although, the anterior and posterior PCs differ in their functions, they share common developmental regimes (Grammont and Irvine 2001, Besse and Pret 2003, Torres *et al.* 2003). These observations demonstrate that *Wolbachia* tropism to the PCs is determined by factors common to both the populations of PCs most likely during their specification.

3.5 *Wolbachia* accumulate in PCs only after PC lineage specification

Using FasIII as a PC marker, we were able to characterize *Wolbachia* tropism to the PCs. However, FasIII immunostaining can identify PCs unequivocally only at stage 4 and beyond. It is not clear at what stage of PC maturation *Wolbachia* accumulate in PCs. Here we addressed this question by two methods.

Firstly, we assessed *Wolbachia* levels in the stalk cells (SC), which form a narrow stem connecting adjacent egg chambers (King 1970, Wu *et al.* 2008). The stalk cells are specified when egg chambers exit the germarium in stage 2 of oogenesis. SCs and PCs derive from the same precursor population cells that is separated from the lateral follicle progenitors (see Fig. 3.14B,H) (Tworoger *et al.* 1999, Chang *et al.* 2013). From the PC/SC precursors, one population differentiates to PC progenitors and the other into SC progenitors (see Fig. 3.14H) (Tworoger *et al.* 1999, Chang *et al.* 2013). We hypothesize that if *Wolbachia* infect only the PC progenitors, we would expect no or little accumulation

of bacteria in the stalk cells. Upon quantifying *Wolbachia* levels in the SC, we found that only a small proportion of SC had any *Wolbachia* infection at all (Fig. 3.11). In *w*Mel and *w*MelCS strains, only 28% (7/25) and 36% (9/25) of SC had *Wolbachia* (Fig. 3.11J). This contrasts with 100% of PCs containing *Wolbachia* in *w*Mel (144/144) and *w*MelCS (201/201). *w*MelPop infected flies had a relatively higher proportion (68%, 31/45, Fig. 3.11J) of infected SC. However, even among the infected SC, we observed a low *Wolbachia* density as compared to PCs (Fig. 3.11D-F). Only in rare instances we found SC with *Wolbachia* levels comparable to that observed in PCs (Fig. 3.11G-I). Furthermore, the frequency of infected lateral follicle cells was consistently lower than the frequency of infected PCs, which was 100% (179/179). These data suggest that *Wolbachia* do not accumulate in precursor cell population common to SC and PC. Our data suggest that *Wolbachia* accumulate in PC precursors after they separate from the common PC/SC precursor (Fig. 3.14H).

We confirmed this possibility by quantifying *Wolbachia* density in the common PC/SC precursors in the germarium. The follicle cells between regions 2b and 3 are thought to be common precursors of both PCs and SCs (yellow cells in Fig. 3.12B) (Larkin *et al.* 1996, Tworoger *et al.* 1999, Bai and Montell 2002, Chang *et al.* 2013). We found no particular group of cells within this region that had particularly high *Wolbachia* accumulation (Fig. 3.12C-C'). Moreover, we compared *Wolbachia* density in the region 2b follicle cells (yellow dashed line in Fig. 3.12D'') to lateral follicle cells (green dashed line in Fig. 3.12D'') and observed no enrichment of *Wolbachia* in region 2b for all three strains tested (Fig. 3.12I). Moreover, the proportion of *Wolbachia* infected cells was

equivalent between regions 2b and 3 of the germarium (Fig. 3.12H). We observed a few instances of early egg chambers that had a matured posterior PCs (as marked by high FasIII expression, Fig. 3.12E'-E") but not the anterior PC. In these, we observed that *Wolbachia* had already accumulated in the posterior PCs suggesting that PCs specification triggers *Wolbachia* intracellular growth.

Furthermore, we used another method to identify PC/SC precursors in the germarium: loss of Eyes Absent (Eya) expression is considered one of the first markers of PC/SC lineage in the germarium (Bai and Montell 2002, Chang *et al.* 2013). We identified cells in region 2b of the germarium that had markedly reduced Eya expression compared to lateral follicle cells in region 3 (Fig. 3.12G-G''). Quantification of *Wolbachia* density in Eya lacking cells (yellow dashed line in Fig. 3.12G') compared to Eya expressing cells (green dashed lines in Fig. 3.12G'') showed no enrichment of *Wolbachia* in these putative PC/SC precursors (Fig. 3.12J) corroborating that *Wolbachia* accumulate in the PCs only after their specification.

To further test our hypothesis, we investigated if a PC fate is sufficient to drive high *Wolbachia* density. Using transgenic flies, we induced ectopic PCs. Eyes absent (Eya) is a PC repressor and its knockdown is sufficient to induce a PC fate (Bai and Montell 2002). We expressed *eya*RNAi under the control of *GR1*-Gal4, a follicle cell driver (Gupta and Schupbach 2003, Goentoro *et al.* 2006) that is expressed beginning in stage 3 of oogenesis (Fig. 3.13A) (supplementary figure 3 in Etchegaray *et al.* 2012). We observed multiple FasIII positive ectopic PCs in stages 5 and 8. We found that every ectopic PC observed

was infected with *Wolbachia* both in stage 5 (23/23, Fig. 3.13B-B") and stage 8 (33/33, Fig. 3.13C-C") egg chambers. In contrast, not every follicle cell is infected with *Wolbachia* (Fig. 3.13B,C). Upon quantification, we found that *Wolbachia* density in these ectopic PCs was comparable to *Wolbachia* density in the normal PCs in the respective stages (Fig. 3.13D). Taken together, these findings demonstrate that a PC fate is essential for *Wolbachia* to accumulate at high levels, and *Wolbachia* accumulate after PC specification from the common polar cell/stalk cell precursor (see Fig. 3.14H).

3.6 *Wolbachia* PC accumulation is most likely through over-replication

Next, we wanted to investigate whether *Wolbachia* accumulate by over-replication in the PCs or by uptake from surrounding FCs. To answer this question, we analyzed the egg chambers that had ectopic PC. We observed large populations of ectopic PCs in each egg chamber analyzed. The density of *Wolbachia* in ectopic PCs was equivalent to normal PCs (Fig. 3.13C), however the ectopic PCs had a substantially increased volume compared to the normal PCs in both stage 5 (~13-fold) and stage 8 (~6.4-fold) egg chambers (Fig. 3.13E). To account for the additional bacteria, *Wolbachia* either over-replicate in the PCs or migrate from the surrounding FCs. As the *Wolbachia* in the PCs are tightly packed in clumps containing several bacteria, it is challenging to count individuals or visualize dividing bacteria. Therefore, we investigated the second possibility by analyzing *Wolbachia* accumulation in the surrounding follicle cells. If *Wolbachia* growth in the PCs is due to uptake from the surrounding FCs, we would expect a depletion of *Wolbachia* in the FCs surrounding the ectopic PCs as compared to the FCs surrounding the normal PCs. Upon comparing these values, we found that the FCs surrounding the ectopic PCs have

comparable density as the FCs surrounding the normal PCs in the corresponding stages (Fig. 3.13F). These findings suggest that *Wolbachia* accumulate in PCs due to over-replication and not due to uptake from surrounding FCs.

3.7 Discussion

The metagenomics revolution revealed that a vast majority of metazoans are colonized by diverse populations of microorganisms. These microscopic partners affect several aspects of the metazoan host biology, including evolution, modulation of immune responses, nutrition, physiology and development (reviewed byMcFall-Ngai *et al.* 2013). Usually animal development is viewed as an autonomous process, in which the metazoan genome drives morphogenetic events. However, there is an exponential growth in literature showing that colonization of specific tissues by environmental bacteria shape host development (Fraune and Bosch 2010). For instance, in the absence of the microbiome, mice gut and Zebrafish fins do not undergo proper development (Stappenbeck *et al.* 2002, Rawls *et al.* 2004). In squids, light organs also do not form in the absence of microbes (McFall-Ngai and Ruby 1991). Furthermore, there are several examples, of bacteria that are maternally transmitted rather than environmentally acquired. In many cases, vertically transmitted bacteria establish an obligatory symbiotic association. For instance, in the absence of *Buchnera* bacteria, aphids lack the nutritional requirements to complete normal development and reproduction (Koga *et al.* 2007).

The influence of bacteria in host development is clearly evident for the intracellular bacteria *Wolbachia*. *Wolbachia*, one of the most common symbionts in arthropods, affect

several aspects of host development and reproduction. In cases where *Wolbachia* symbiosis is obligatory, host development depends on the presence of the bacteria. For instance, the wasp *Asobara tabida* requires *Wolbachia* for completion of oogenesis and egg maturation (Dedeine *et al.* 2005). Several filarial nematodes also require *Wolbachia* for successful reproduction. In the absence of *Wolbachia*, embryonic and larval development are impaired (reviewed bySlatko *et al.* 2010).

Despite extensive evidence of *Wolbachia* affecting host development and reproduction, the converse aspect of this interaction, that is, how host development affects *Wolbachia* intracellular growth is less well established. In the literature, there are few examples of coordination of *Wolbachia* growth with host development: In certain mosquito species, when adverse environmental conditions suspend embryonic development and eggs enter diapause, *Wolbachia* levels are reduced accordingly (Ruang-areerate *et al.* 2004). Another remarkable example is the tortuous path that *Wolbachia* undertake to infect the female germline in filarial nematodes. In several species of the *Onchocercidae* family, *Wolbachia* are first concentrated in the precursors cells that form the germline and hypodermal lineages. Surprisingly, in the next mitosis, *Wolbachia* are excluded from the germline precursors. Later in development they invade the germline from the distal tip cells, the worm equivalent of the germline stem cell niche (Landmann *et al.* 2012). *Wolbachia* tropism to these hypodermal progenitor cells and the stem cell niche is essential for transmission to the next generation.

In the *Drosophila* genus, *Wolbachia* have tropism to stem cell niches. In fact, tropism to the ovarian somatic stem cell niche is ubiquitous in all *Drosophila* species tested and contributes in increasing *Wolbachia* titers in the germline (Toomey *et al.* 2013). However, in this system, to determine the kinetics of infection from SSCN progenitor cells to ovary maturation is challenging. Unlike worms, where the cell lineages have been traced, the precursors of the SSCN in the *Drosophila* ovary are not easily identified. In flies, the SSCN forms during pupal ovary development from a dynamic, non-dedicated population of niche cells, originating from signaling between the germline and maturing stalk cells in the pupae (Sahai-Hernandez and Nystul 2013, Vlachos *et al.* 2015). Here we identify a novel *Wolbachia* tropism to the polar cells (PCs) of *Drosophila*, a cell type that allows us to determine the kinetics of tropism throughout all developmental stages from progenitor cells to the maturation of the cell type.

The PCs are a subset of follicle cells present at either poles of the developing egg chamber and have multiple signaling pathways in common with the stem cell niches, during development (Xi *et al.* 2003, Decotto and Spradling 2005, Le Bras and Van Doren 2006, Wu *et al.* 2008). In a survey across various *Drosophila* species, tropism to the PCs was found in most *Drosophila* species tested and in all stages of PC development (Fig. 3.2). However, one *Wolbachia* strain (*w*Ri) did not have a preferential *Wolbachia* tropism to PCs. We fitted the PC tropism character to *Wolbachia* phylogeny created using multilocus sequence typing (Fig. 3.3) (Paraskevopoulos *et al.* 2006). This analysis shows that the ancestral strains of *Wolbachia* had this characteristic and its loss in *w*Ri occurred most likely during its recent separation from its closest living relative, *w*Sh. *w*Ri has a

highly mosaic genome with a multitude of mobile elements and breakpoints (Klasson *et al.* 2009). It is thought to be one of the most highly recombining intracellular bacterial genomes known to date (Klasson *et al.* 2009). This has led to *w*Ri exhibiting highly variable phenotypes. For instance, *w*Ri infection initially caused a fecundity deficit in *Drosophila simulans* upon recent infection in 1988. However, it evolved within 16 years to give a fecundity benefit by 2004 (Weeks *et al.* 2007). Moreover, it has evolved variable tropism compared to its closest living relative, wSh, to both the germline stem cell niche of the ovaries as well as the hub in the testis (Toomey and Frydman 2014). Considering these findings, it is not surprising that *w*Ri has lost its PC tropism phenotype.

We studied three different *Wolbachia* strains which infect *Drosophila melanogaster* in further detail. We observed that *Wolbachia* PC tropism occurred early in oogenesis (Stage 4) and persisted up until late oogenesis. As egg chambers progress through oogenesis, we observed that *Wolbachia* density in PCs relative to the lateral follicle cells increased constantly. As the egg chamber develops, the number of lateral follicle cells increase from about 50 in stage 4 to around 1000 in stage 6 (Gonzalez-Reyes and St Johnston 1998, Xi *et al.* 2003, Wu *et al.* 2008). However, during this time the number of PCs remain constant (two at each pole). The apparent increase of *Wolbachia* density in PCs could be attributed to the decrease in density in the other follicle cells. While the number of follicle cells increases rapidly about 20-fold, it is possible that *Wolbachia* are unable to replicate fast enough to maintain their density. Whereas in the PCs, it can replicate to a much higher level as the number of cells do not increase. To address this, we compared *Wolbachia* densities in the lateral follicle cells alone across various stages of oogenesis

(Fig. 3.6). This shows that the densities of *Wolbachia* in the follicle cells do not decrease over time. Furthermore, these data also suggest that *Wolbachia* coordinate their intracellular levels with lateral follicle cell development. The *w*Mel and *w*MelCS strains coordinate their growth with lateral follicle cell mitosis until stage 6 and volume increase until stage 10, remaining at constant densities.

However, we found that the density of *w*MelPop in the lateral follicle cells decreased steadily from stage 4 to stage 10 (Fig. 3.6C). In this case, the high density in the PCs could be partially contributed by normalization to lateral follicle cells. However, the quantification of the absolute densities of *Wolbachia* in the PCs shows that a high density is achieved early in oogenesis and those high levels are maintained (Fig. 3.7). *w*MelPop, a pathogenic strain of *Wolbachia,* over-replicates in insect tissues and leads to cell lysis in neuronal cells (Min and Benzer 1997). Previously, we also observed cell lysis of hub cells infected with *w*MelPop (Toomey and Frydman 2014). This pathogenic effect suggest that *w*MelPop has lost its coordination with the host and they keep growing even when the intracellular environment no longer can support their growth. In agreement, our data in the PC, also indicate that *w*MelPop lost their coordination with host cell developmental events (Fig. 3.7).

We observe a substantial increase in *Wolbachia* density between stages 8 and 9 (Fig. 3.5G-I). As there is no increase in follicle cell number or size between these stages, the increase in density is very specific to *Wolbachia* in the PCs, indicating certain signaling pathways specific to PCs to be involved in the regulation of *Wolbachia* density. Calculating

the rate of *Wolbachia* PC accumulation over time further shows that *Wolbachia* increase their intracellular accumulation rapidly between stages 8 and 9 of oogenesis over a comparatively short period of time (Fig. 3.9). At the end of stage 8, the anterior PCs along with 6-8 border cells detach from the epithelium, extend processes in between the nurse cells and migrate through the egg chamber to the anterior border of the oocyte. During this process, the PCs are associated very closely with the germline and provide ample opportunity for *Wolbachia* to traverse into the germline. Previously, Toomey *et al*. had proposed that the *Wolbachia* amplification in the follicular epithelium would be a crucial step for *Wolbachia* to colonize the germline at high titers (Toomey *et al.* 2013). Moreover, in both parasitic worms *Brugia malayi* and *Litomosoides sigmodontis*, *Wolbachia* have been shown to preferentially replicate in the rachis, a central actin-rich structure connecting the distal ovaries, before colonizing newly formed germ cells (Landmann *et al.* 2012). Preferential replication of *Wolbachia* in the PCs could act as one of the amplification steps between *Wolbachia* tropism to the SSCN and the mature germline. In certain *Drosophila* species, *Wolbachia* infection of the germline is inconsistent, wherein certain egg chambers lack *Wolbachia* altogether in their germline, even within individual ovarioles (Casper-Lindley *et al.* 2011). However, all the eggs laid by these females have *Wolbachia* suggesting that *Wolbachia* re-enters the germline from somatic cells present in the egg chamber. In *D. melanogaster*, electron micrographs show *Wolbachia* invading the germline from follicle cells (see FigS10 in Toomey *et al.* 2013). Our data suggest that the stage 9 migration of anterior PCs provides an opportunity for *Wolbachia* transfer from the PCs to the germline. These findings therefore demonstrate that *Wolbachia* coordinate their

replication and intracellular accumulation with specific host developmental events to facilitate their efficient transmission. There is precedence from other systems showing that *Wolbachia* can respond to specific host developmental events. For instance, in the parasitic worm *Brugia malayi*, *Wolbachia* levels remain constant in the microfilaria and larval stages, but the bacterial levels increase significantly within one week of the infection of a mammalian host by the worm (Fenn and Blaxter 2004, McGarry *et al.* 2004, Landmann *et al.* 2010).

If stage 9 migration of PCs is a viable option for *Wolbachia* to enter the germline, it follows that the density in the anterior PCs should increase at a much higher rate than the posterior ones. Some observations do suggest this hypothesis. For instance, *w*Mel has a higher density in stage 8 anterior PCs (just before the migration event) and *w*MelPop has a higher density in stage 9 anterior PCs (during the migration event). However, we observed no significant difference between *Wolbachia* levels in anterior and posterior PCs in other stages of *w*Mel or *w*MelPop infected flies or any oogenesis stage of *w*MelCS infected flies. This indicates that signaling pathways common to both sets of PCs are important for *Wolbachia* accumulation. Among the 1000 lateral follicle cells, only 6-8 border cells migrate. During this transition, the anterior PCs secrete the JAK-STAT cytokine UPD which activates STAT in the border cells and induces them to migrate (Silver and Montell 2001). Apart from this, ecdysone signaling, EGFR signaling, and DE-cadherin/Armadillo mediated cell adhesion are required for proper border cell migration (Oro *et al.* 1992, Niewiadomska *et al.* 1999, Bai *et al.* 2000, Duchek and Rorth 2001, Duchek *et al.* 2001). However, some of them such as JAK-STAT signaling and DE-cadherin/Armadillo

mediated cell adhesion are upregulated even in the posterior follicle cells (Silver and Montell 2001). Further, among the migrating cells, we observe an increased *Wolbachia* level specifically in the polar cells and not in the border cells (Fig. 3.14 and Fig. 3.8A",B") indicating that *Wolbachia* tropism to the polar cells is highly specific in nature. Future studies will aim at characterizing signaling pathways that would be important for this stage specific increase in *Wolbachia* titers.

PCs are determined very early in egg chamber morphogenesis. Somatic stem cells in the germarium divide and undergo transient amplification to give rise to all the follicle cells surrounding the germline. Unlike lateral follicle cells which keep dividing until stage 6 of oogenesis, the PCs and SCs cease division as soon as they are specified in the germarium. (Gonzalez-Reyes and St Johnston 1998, Roth 2001, Torres *et al.* 2003, Assa-Kunik *et al.* 2007, Wu *et al.* 2008). The PCs and SCs are thought to arise from the same precursor population (Larkin *et al.* 1996, Tworoger *et al.* 1999). Recently, Chang *et al.* corroborated the presence of PC/SC precursors by showing the expression of Castor, a zinc finger protein, in the presumptive PC/SC precursor population in region 2b of the germarium (Chang *et al.* 2013). Considering these findings, if *Wolbachia* accumulate highly in these PC/SC precursor population, we should find similar levels of *Wolbachia* in the stalk cells. However, when investigated, we found that only a small proportion of *w*Mel (29%, Fig. 3.11J), and *w*MelCS (35%, Fig. 3.11J) of stalks have any *Wolbachia* at all (see Fig. 3.11A-C). *w*MelPop, unsurprisingly, infected about 70% of stalks (Fig. 3.11J) but the infection density was rarely as high as a PC infection (Fig. 3.11D-F). The PC/SC precursors reside in the region 2b of the germarium (Larkin *et al.* 1996, Tworoger *et al.* 1999, Bai and

Montell 2002, Chang *et al.* 2013). Although the exact separation point of PC and SC lineages is not clear, if *Wolbachia* are already enriched in the PC precursors, we might observe a few cells with high bacterial density compared to the lateral follicle cells in region 3. However, we observed no single cell/group of cells which consistently had a preferential accumulation of *Wolbachia* in this region. Moreover, the overall density of *Wolbachia* in these cells was comparable to that in the lateral follicle cells (Fig. 3.12G). The putative PC/SC precursors in region 2b can also be identified by a loss of Eya expression (Bai and Montell 2002, Chang *et al.* 2013). We observed no specific *Wolbachia* accumulation to these putative PC/SC precursor population (Fig. 3.12G-G''). We also observed certain egg chambers with matured posterior anterior PCs (Fig. 3.12E-E'''). In these, we observed *Wolbachia* preferential accumulation in the matured PCs but not in the precursor populations including the SC precursors. These results implicate that *Wolbachia* preferentially accumulate in PCs after differentiation of the PC lineage from the common PC/SC lineage.

To further distinguish the lineage specificity of *Wolbachia* accumulation, we induced ectopic PCs by expressing eyaRNAi under the control of a *GR1*-Gal4 driver. Knockdown of Eya, a known PC fate repressor, led to the induction of multiple FasIII positive ectopic PCs (Bai and Montell 2002, Grammont and Irvine 2002). GR1-drives gene expression beginning stage 3 of oogenesis (see supplementary figure 3 Etchegaray *et al.* 2012) and consistent with that we found a majority of ectopic PCs in stages 5 and 8 of oogenesis. As the ectopic PCs were induced after stage 3, we can be certain that these cells had assumed lateral follicle cell fate prior to becoming PCs. *Wolbachia* were found to infect

every ectopic PCs observed at a high density (Fig. 3.13B,C). In contrast, many lateral follicle cells lack *Wolbachia* altogether, suggesting PC fate specification is sufficient to drive *Wolbachia* tropism.

Our results also suggest that *Wolbachia* accumulate in the PCs due to over-replication. Upon eyaRNAi, we observe large populations of ectopic PCs in both stage 5 and stage 8 egg chambers with densities equivalent to the normal PCs. The volume occupied by these ectopic PCs is 6 to 13-fold larger than the corresponding normal PCs. This means that there are about 6 to 13-fold more *Wolbachia* in the ectopic PCs compared to the normal PCs. If *Wolbachia* from the surrounding FCs migrate to the PCs to make up for this increase, we would expect the FCs surrounding ectopic PCs to have a much lower *Wolbachia* density compared to the FCs surrounding the normal PCs. However, we observe no difference in *Wolbachia* between FCs surrounding the ectopic PCs and the FCs surrounding the normal PCs. Taken together, these results suggest that the mechanism of *Wolbachia* growth in PCs is over-replication in the PCs and not migration from the surrounding lateral FCs, although uptake from other neighboring cells can't be completely ruled out.

To summarize our findings, we propose a model (Fig. 3.14) for *Wolbachia* accumulation in the PCs during development. As somatic stem cells (SSC) divide in the germarium, their progeny assume either a polar/stalk cell precursor fate or lateral follicle cell fate (Fig. 3.14B,C). Around stage 2 of oogenesis, the polar/stalk cell precursors differentiate into PCs (red in Fig. 3.14D,D') or stalk cells (pink in Fig. 3.14D,D').

Wolbachia start accumulating in the PCs once the separation of the polar and stalk cell precursors occurs (Fig. 3.14H). When oogenesis progresses further, between stage 4 and 8, *Wolbachia* (green dots in Fig. 3.14D',E') divide rapidly to increase their intracellular levels in the PCs specifically whereas the *Wolbachia* levels in the lateral follicle cells (grey) remains constant. Between stage 8 and 9 (Fig. 3.14E-G'), *Wolbachia* levels increase rapidly in PCs. At stage 9, the anterior PCs migrate through the germline allowing for a perfect opportunity for *Wolbachia* to traverse into the germline (Fig. 3.14F,F'). Also, high *Wolbachia* density in the posterior PCs at this stage means *Wolbachia* can traverse directly into the oocyte (Fig. 3.14G,G').

Being maternally transmitted, *Wolbachia* need to colonize the germ cells at high densities. However, in many hosts, *Wolbachia* end up colonizing other somatic cell types at high densities (Frydman *et al.* 2006, Landmann *et al.* 2012, Toomey *et al.* 2013, Toomey and Frydman 2014) and then migrate to the germline during its maturation. It has been challenging to study *Wolbachia* accumulation to these somatic cell types due to several reasons. Here we describe a novel *Wolbachia* tropism to the PCs of the *Drosophila* ovary – a molecularly well characterized system which will be extremely beneficial to study the molecular mechanisms of *Wolbachia* tropism to somatic tissues of the host. It is vital for symbionts to coordinate their accumulation with host developmental events and keep their replication in check so that they do not harm the host. *Wolbachia* have been shown to be able to sense certain host developmental cues and coordinate their accumulation (Landmann *et al.* 2010). Here we demonstrate that even in the PCs, *Wolbachia* are able to coordinate their replication with specific host developmental events and accumulate to very

high densities. Overall, we have demonstrated a novel *Wolbachia* tropism to the PCs of *Drosophila* ovaries. Further studies of the mechanisms of this tropism will shed light on the molecular cues which *Wolbachia* utilize to target specific cells in the host and sense host developmental events to coordinate their high intracellular accumulation.

Figure 3.1 *Wolbachia* tropism to stem cell niches in *Drosophila* gonads.

(A) Schematic of a *Drosophila* germarium showing germline stem cells (GSC) in red, germline stem cell niche (GSCN) in green (with a red bracket), somatic stem cells (SSC) in blue and the somatic stem cell niche in green (with red arrows). (B) *Wolbachia* (green) has tropism to the GSCN (marked with a red bracket). (C) *Wolbachia* (green) has tropism to the SSCN (marked by red arrows). (D) Schematic of a *Drosophila* testis hub with cell nuclei in blue. The germline stem cells (GSCs, grey) and cyst stem cells (CySCs, white) reside at the hub (red). (E) *Wolbachia* (green) tropism to the hub (hub, labeled by Armadillo (Arm) staining in red).

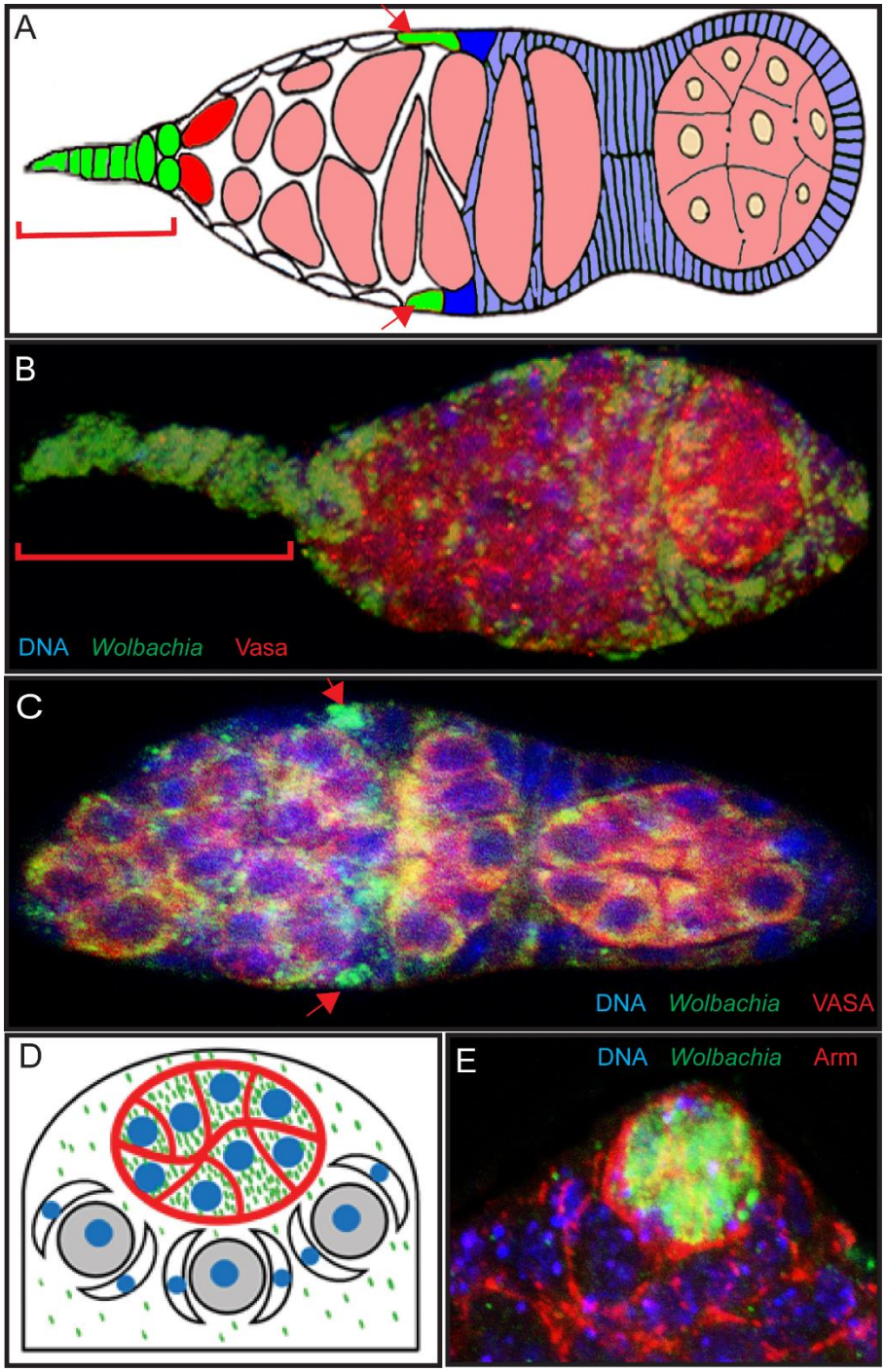

A
B
DNA Wolbachia Vasa
C
DNA Wolbachia VASA
D
E
DNA Wolbachia Arm

Figure 3.2: *Wolbachia* tropism to the polar cells (PCs) is evolutionarily conserved across the *Drosophila* genus.

(A) A pair of PCs is located at the anterior and posterior poles of the egg chamber. PCs (shown in red), are part of the follicular epithelium (dark grey) that surrounds the germline (light grey). (B–K) Confocal images of 10 different *Wolbachia* strains (strains indicated in the bottom right corner) infecting egg chambers of the respective *Drosophila* native species (indicated in the bottom left corner). The levels of *Wolbachia* (*Wolbachia* 16S rRNA labeled in green) in the germline are usually higher than the surrounding follicular epithelium, with exception of the PCs (labeled by FasIII in red). Higher magnification of the PCs is shown below the respective egg chamber in the color overlay (‘) or only the *Wolbachia* channel in grey (“). (L) *Wolbachia* density in the PCs were calculated relative to the follicular epithelium. In every *Drosophila*-*Wolbachia* pair (except for *Dsim w*Ri) we find that the *Wolbachia* density is higher in the PCs compared to the other follicle cells. N≥20 for each species. Scale bars = 10μm. Error bars show s.e.m.

A
Polar Cells
Follicle Cells
B
D. melanogaster wMelPOP
B'
B''
C
D. melanogaster wMelCS
C'
C''
D
D. simulans wNo
D'
D''
E
D. mauritiana wMau
E'
E''
F
D. yakuba wYak
F'
F''
G
D. teisseri wTei
G'
G''
H
D. tropicalis wWil
H'
H''
I
D. melanogaster wMel
I'
I''
J
D. sechellia wSh
J'
J''
K
D. simulans wRi
K'
K''
L
Dmel wMelPOP
Dmel wMelCS
Dsim wNo
Dmau wMau
Dyak wYak
Dtei wTei
Dtrop wWil
Dmel wMel
Dsech wSech
Dsim wRi
0
2
4
6
8
Relative PC Wolbachia density

Figure 3.3: Polar cell tropism fit to *Wolbachia* phylogeny.

Polar cell tropism fit to the *Wolbachia* phylogeny using MacClade software (Maddison and Maddison 1989). All species of *Wolbachia* except for *w*Ri exhibit tropism to the polar cells. *Wolbachia* phylogeny and Bayesian posterior probabilities assigned to the branches based on multilocus sequence typing (Paraskevopoulos *et al.* 2006). The lengths of branches shown here are arbitrary and not representative of phylogenetic distance.

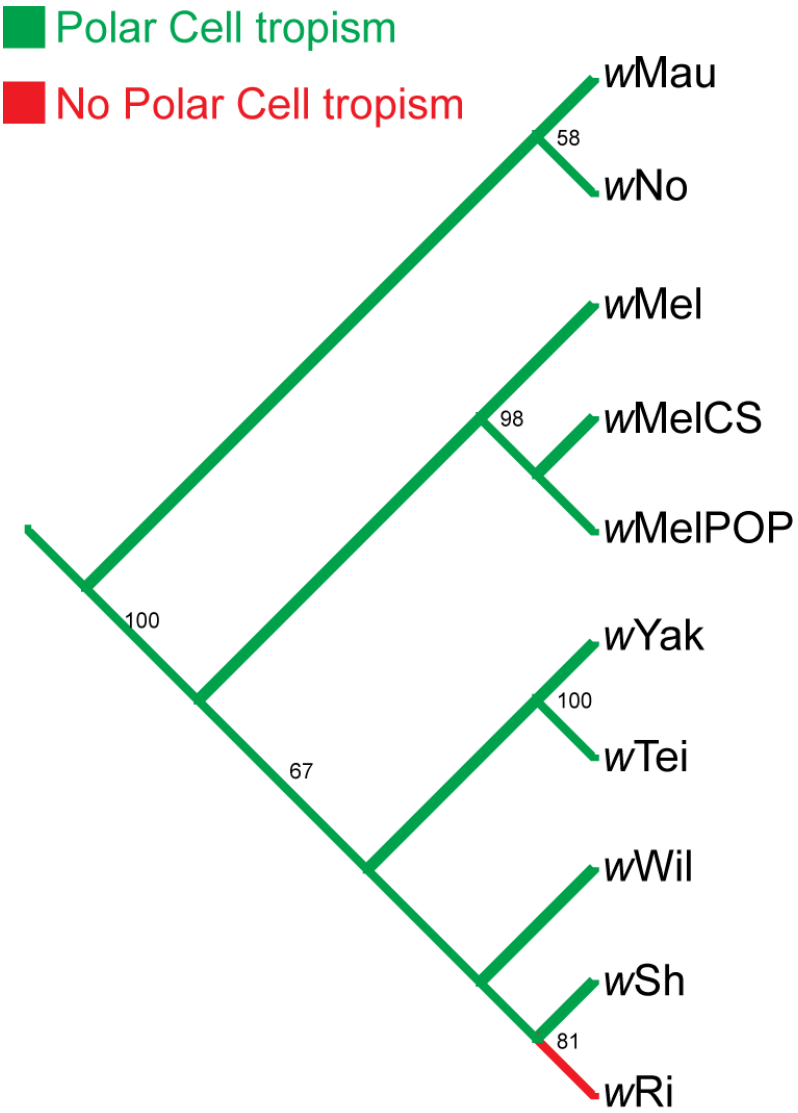


Figure 3.4: *Wolbachia* does not accumulate in putative polar cells in *Culex pipiens*.

(A) *Culex pipiens* egg chamber labeled with DNA in blue, N-cadherin in red and *Wolbachia* in green. Follicle cells expressing a high level of N-cadherin were considered putative polar cells (white arrowheads). Like *Drosophila*, a pair of polar cells localized at both poles of the egg chamber were identified. These cells had no *Wolbachia* accumulation (*Wolbachia* channel in (C)).

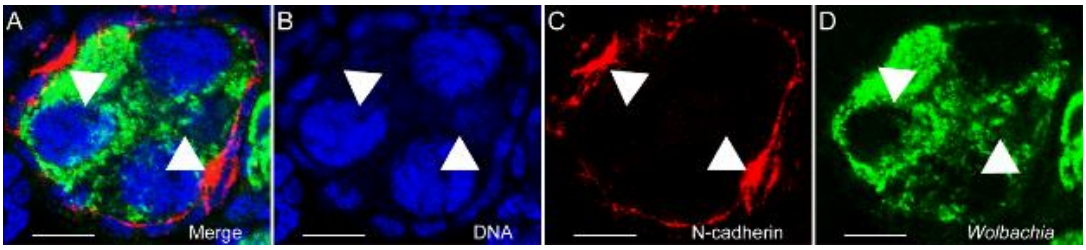


Figure 3.5: PC *Wolbachia* density increases with stages of oogenesis.

(A) Schematic of *Drosophila* oogenesis encompassing 14 stages of oogenesis shows the progression of the germline, shaded in light grey, from stem cell to a mature egg. There is a layer of somatic follicle cells, in dark grey, surrounding the germline in every stage. A pair of PCs (PC), shaded in Red, is present at either pole of the egg chamber. Confocal images of various stages of oogenesis, St4 (B), St5 (C), St8 (D), St9 (E) and St10 (F) show that at every stage, there is a high accumulation of *Wolbachia* (*w*MelPop, green) in the PCs (labeled by FasIII, red) compared to the surrounding follicular epithelia. Upon quantification, *w*Mel (G), *w*MelCS (H), *w*MelPOP (I), we find that *Wolbachia* density in the PC, normalized to surrounding follicle cells, increases with the stages of oogenesis with a particularly (and conserved) sharp increase between stages 8 and 9. *Wolbachia* labeled by 16SrRNA FISH. N≥20 for each stage. Scale bars = 10μm. Error bars show s.e.m. *p<0.05, **p<0.01, ***p<0.001, Students t test.

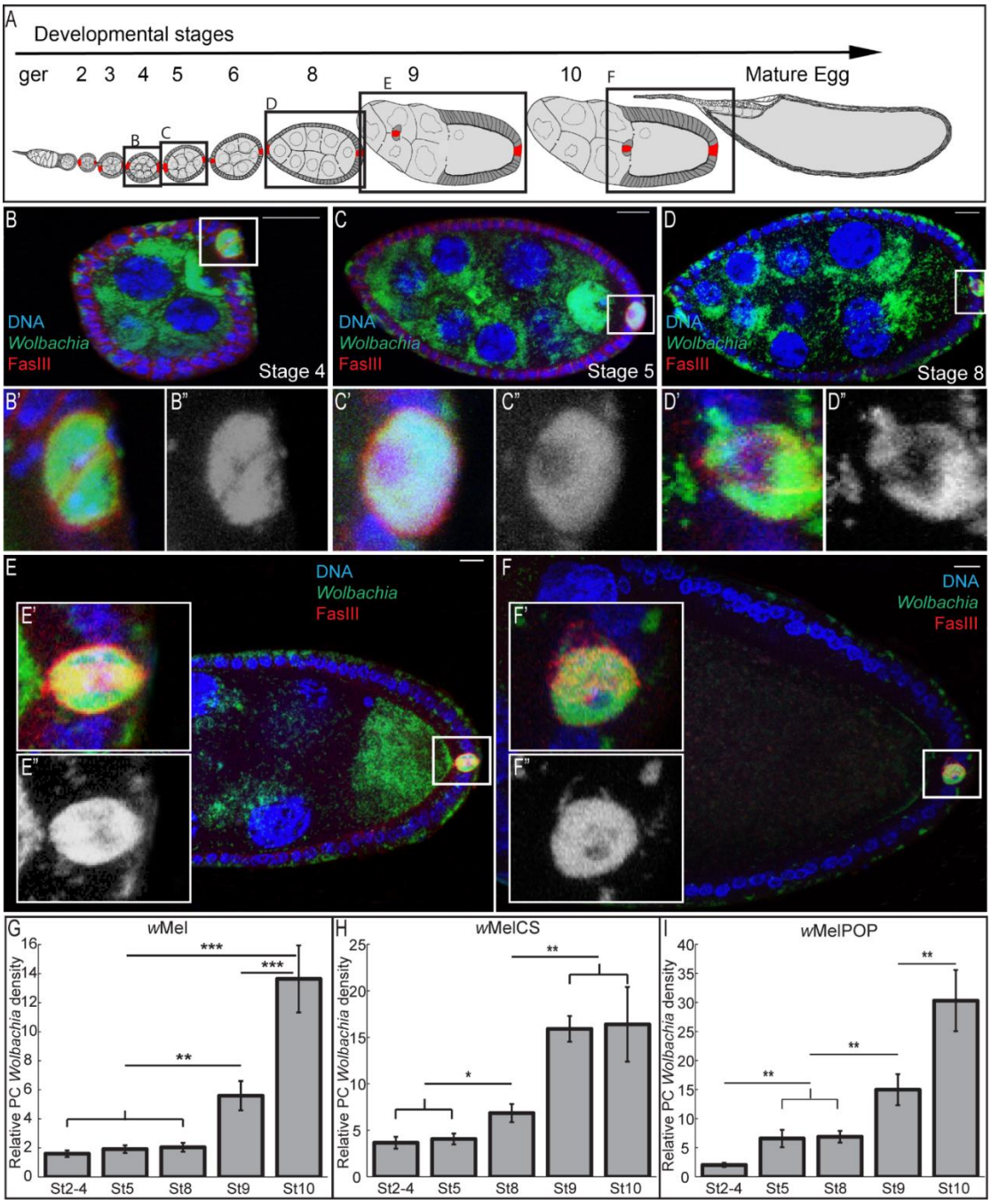

A
Developmental stages
ger 2 3 4 5 6 8 9 10 Mature Egg
B C D E F
B
DNA
Wolbachia
FasIII
Stage 4
C
DNA
Wolbachia
FasIII
Stage 5
D
DNA
Wolbachia
FasIII
Stage 8
B' B" C' C" D' D"
E
DNA
Wolbachia
FasIII
E' E"
F
DNA
Wolbachia
FasIII
F' F"
G
wMel
Relative PC Wolbachia density
St2-4 St5 St8 St9 St10
H
wMelCS
Relative PC Wolbachia density
St2-4 St5 St8 St9 St10
I
wMelPOP
Relative PC Wolbachia density
St2-4 St5 St8 St9 St10

Figure 3.6: *Wolbachia* density in the lateral follicle cells.

Quantification of *Wolbachia* density in the lateral follicle cells alone shows that there is no increase in *Wolbachia* density as egg chambers progress through oogenesis for *w*Mel (A) or *w*MelCS (B). However, the *Wolbachia* density in the *w*MelPop (C) infected flies keeps decreases steadily as the egg chambers progress through oogenesis. Note that the *Wolbachia* density of *w*MelPop in lateral follicle cells is an order of magnitude higher than *w*Mel or *w*MelCS. At these densities, there is most likely a breakdown of mutualism. a,b,c,d denote statistically distinct groups, Student's t test. N>20 for each stage.

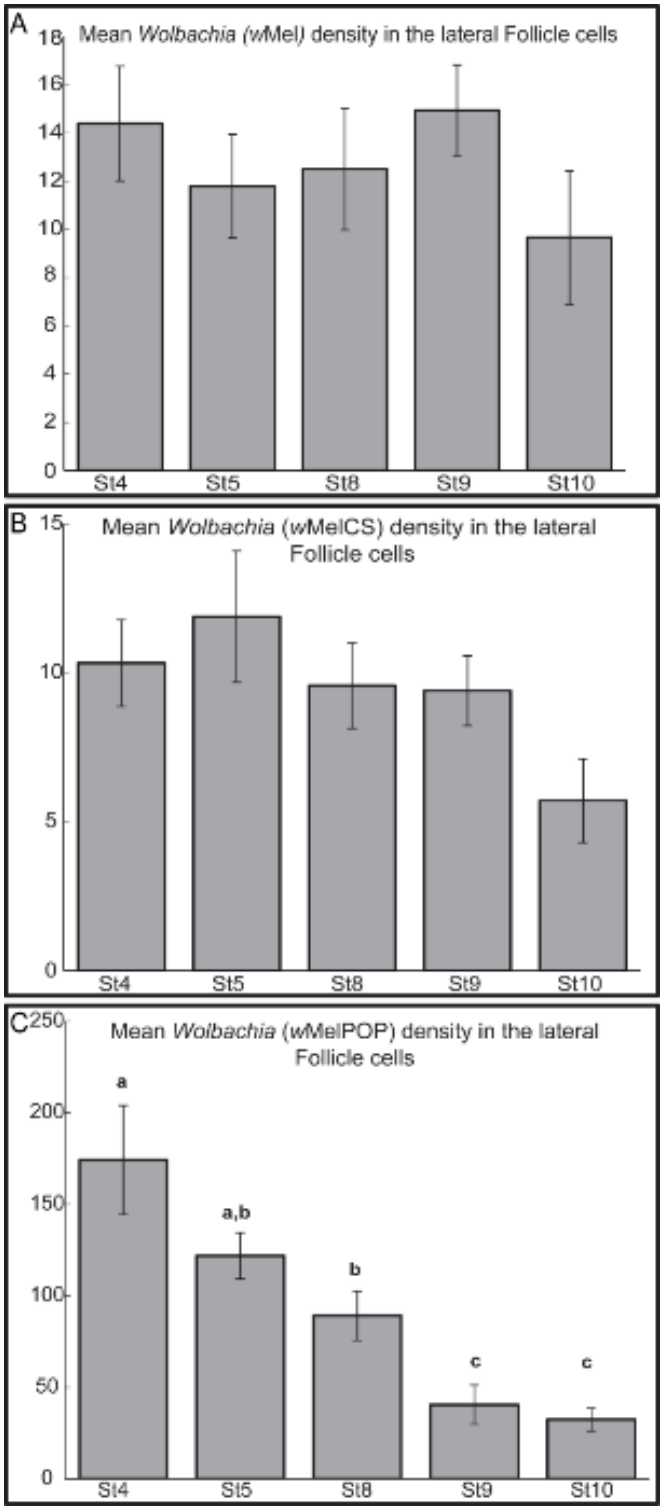

A
Mean Wolbachia (wMel) density in the lateral Follicle cells
18
16
14
12
10
8
6
4
2
0
St4
St5
St8
St9
St10
B
15
Mean Wolbachia (wMelCS) density in the lateral Follicle cells
10
5
0
St4
St5
St8
St9
St10
C
250
Mean Wolbachia (wMelPOP) density in the lateral Follicle cells
a
200
a,b
150
b
100
c
c
50
0
St4
St5
St8
St9
St10

Figure 3.7: *w*MelPop achieve high densities in polar cells.

*w*MelPop density in the polar cells plotted without normalizing to the lateral follicle cells shows that they achieve high densities in the polar cells early in oogenesis and maintain these levels till stage 10. N>20 for each stage.

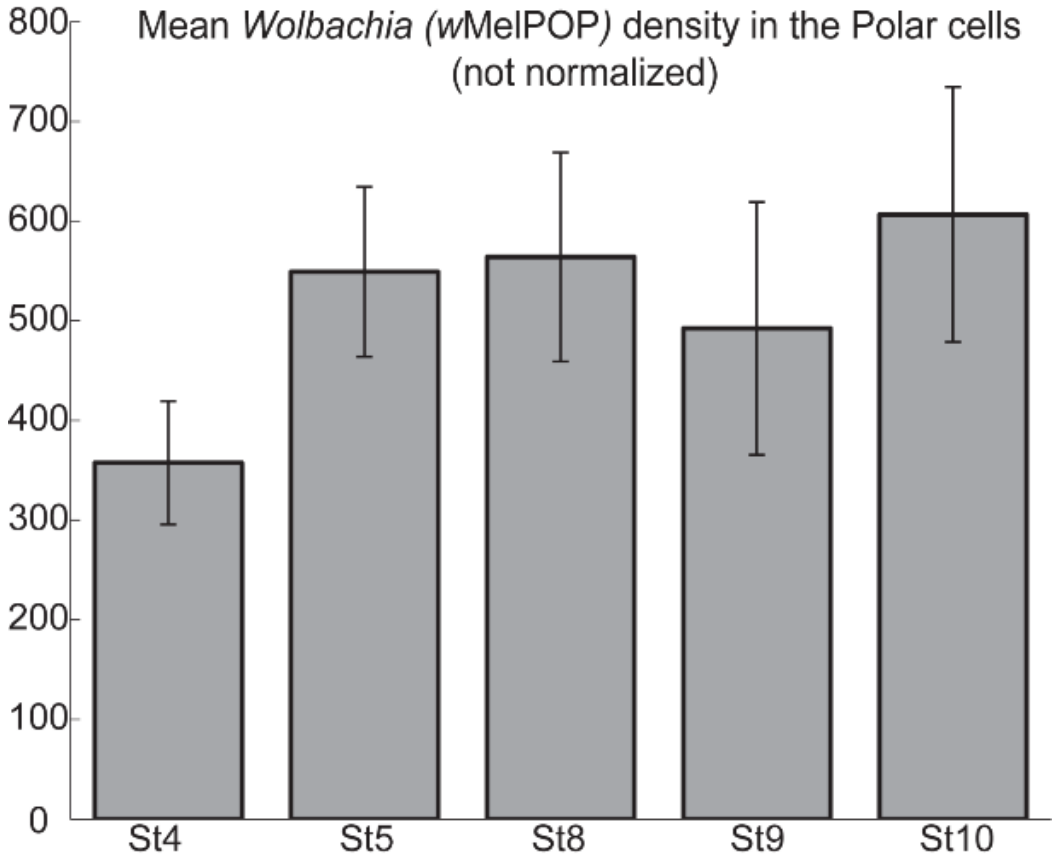


Figure 3.8: *Wolbachia* in migrating anterior polar cells of stage 9.

The anterior polar cells migrate through the germline in stage 9. At both early (A) and late (B) stages of migration, we observe an elevated *Wolbachia* level (green) in the polar cells compared to the follicle cells. Insets (A',B') show a closeup of the anterior polar cells, (A",B") show *Wolbachia* channels alone. Note that in the anterior migrating polar cells (red dashed line), the *Wolbachia* density is higher than the border cells (blue dashed line) and even the surrounding germline (B-B").

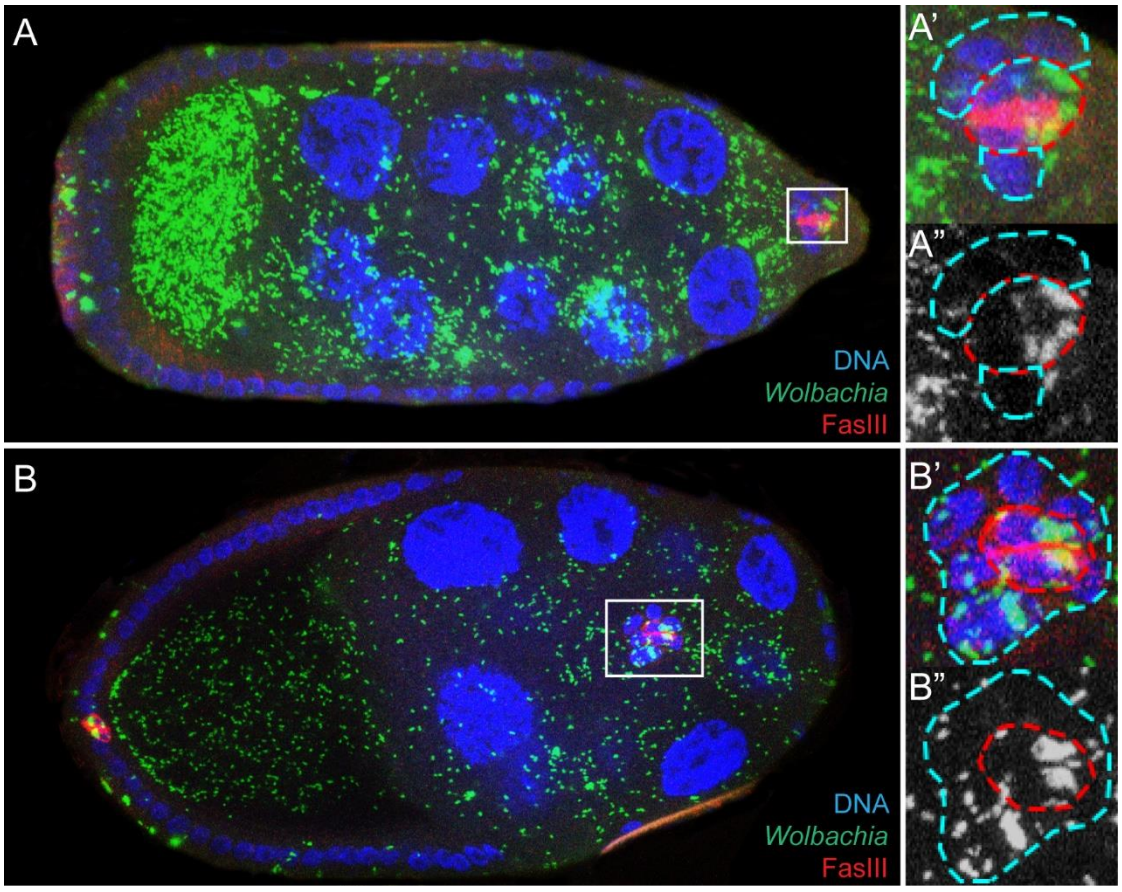


Figure 3.9: *Wolbachia* density increases rapidly between stages 8 and 10.

Quantification of relative *Wolbachia* density in the polar cells shows that *Wolbachia* density doesn't increase linearly with time. In all 3 *Wolbachia* strains tested, (A) *w*Mel, (B) *w*MelCS, and (C) *w*MelPop, *Wolbachia* density increases rapidly between stages 8 and 10 over a period of 15 hours. However, the increase in density is not significant between stages 4 and 8 over a period of about 25 hours. Timing of egg chambers was adapted from Lin and Spradling (David 1968, Lin and Spradling 1993)

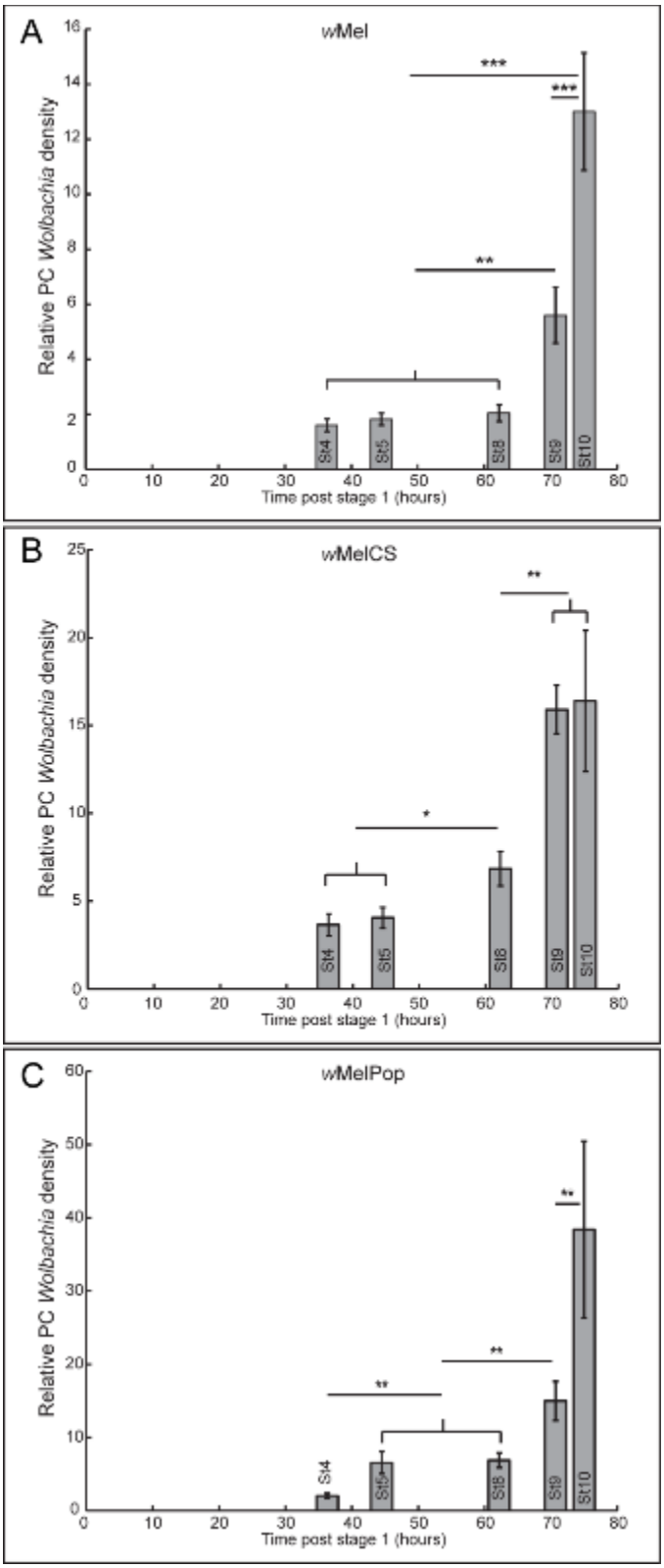

A
wMel
Relative PC Wolbachia density
Time post stage 1 (hours)
St4
St5
St8
St9
St10
B
wMelCS
C
wMelPop

Figure 3.10: *Wolbachia* accumulation in anterior and posterior PCs is equivalent.

(A) Stage 9 and (D) Stage 10 egg chambers showing similar *Wolbachia* density in both the anterior and posterior PCs. Insets (B-B") and (E-E") show a migrating anterior PCs with a very high *Wolbachia* accumulation. Note that the *Wolbachia* density (*w*MelPop, green) in the PCs (red dashed line) is more than the border cells (blue dashed line) and even the surrounding germline. Posterior PCs insets (C-C") and (F-F") show similar accumulation of *Wolbachia* (*w*MelPop, green) as the anterior PCs. (G-I) Quantification shows that in most stages of oogenesis tested, *Wolbachia* density in the anterior and posterior PCs are equivalent. *Wolbachia* labeled by 16SrRNA FISH. N≥20 for each stage. Scale bars = 10μm. Error bars show s.e.m. *$p<0.05$, Student's t test.

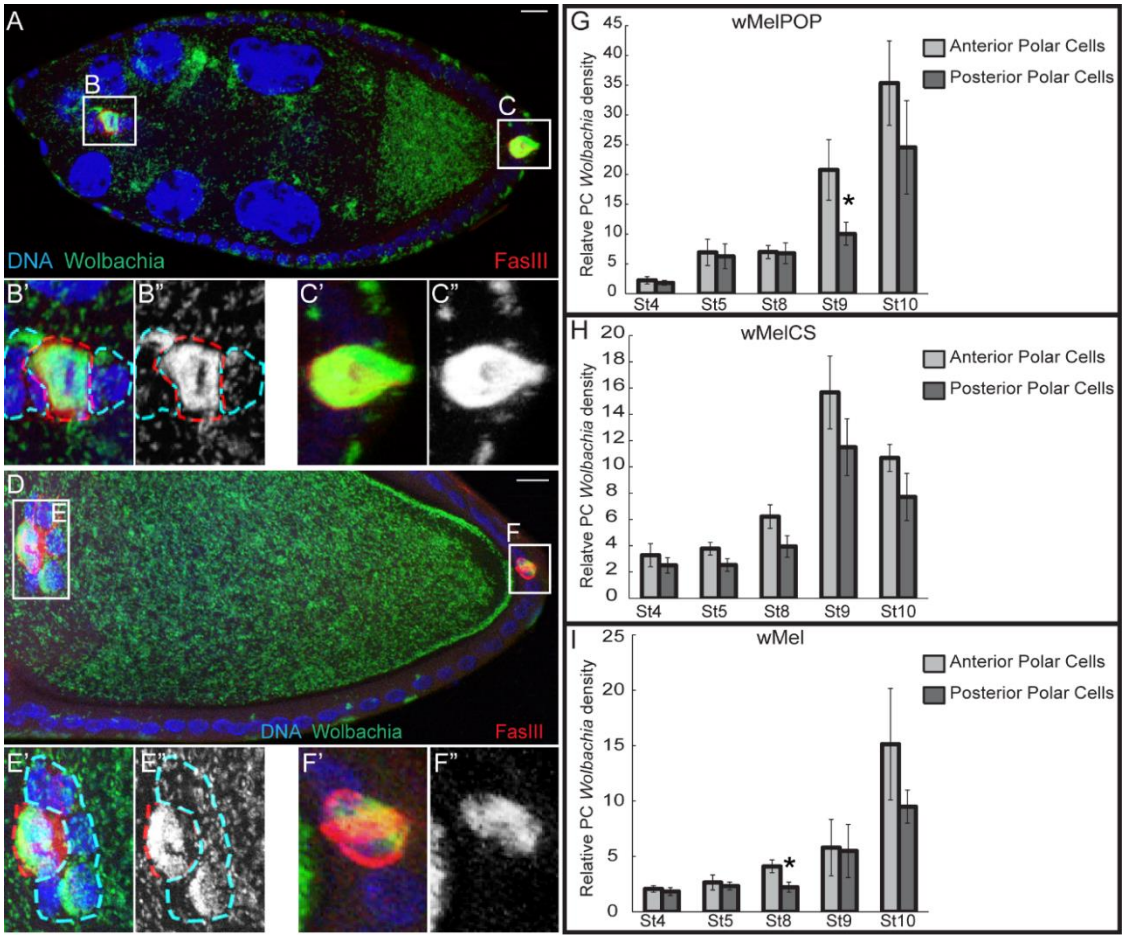

A
B
C
DNA Wolbachia
FasIII
B'
B"
C'
C"
D
E
F
DNA Wolbachia
FasIII
E'
E"
F'
F"
G
wMelPOP
Anterior Polar Cells
Posterior Polar Cells
Relative PC Wolbachia density
St4
St5
St8
St9
St10
H
wMelCS
Anterior Polar Cells
Posterior Polar Cells
Relative PC Wolbachia density
I
wMel
Anterior Polar Cells
Posterior Polar Cells
Relative PC Wolbachia density

Figure 3.11: *Wolbachia* accumulation in the stalk cells is lower than the PCs.

(A) Two egg chambers connected by a stalk. (B-B") Stalk cells are not infected with *Wolbachia* (wMelPop, green) whereas the PCs (C-C") contain an elevated level of *Wolbachia*. (D) An egg chamber connected by a stalk which has low level of *Wolbachia* infection. Stalk cell insets (E-E") and PC insets (F-F") show that in stalk cells infected with *Wolbachia*, the levels of *Wolbachia* are much lower than those in the PCs. (G) In rare cases, *Wolbachia* accumulate in the stalk cells at a level comparable to the PCs. Stalk cell insets (H'-H") and PC insets (I'-I") show comparable levels of *Wolbachia* intracellular accumulation. Quantification (J) shows the percentage of stalk cells which have any *Wolbachia* infection (like D) in all 3 strains of *Wolbachia* tested. *Wolbachia* labeled by 16SrRNA FISH. Error bars show 95% c.i. Scale bars =10μm. N≥20 stalk cells for each *Wolbachia* strain. *$p<0.05$, Two proportions Z test.

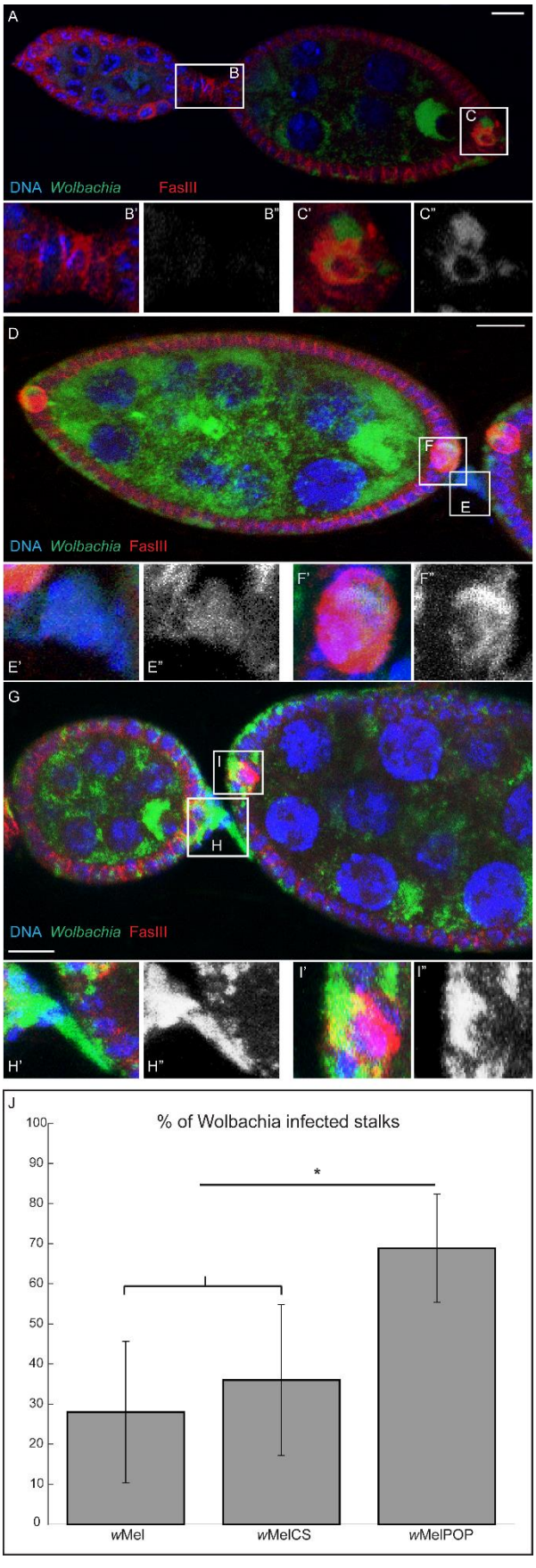

A
B
C
DNA Wolbachia FasIII
B'
B''
C'
C''
D
F
E
DNA Wolbachia FasIII
E'
E''
F'
F''
G
I
H
DNA Wolbachia FasIII
H'
H''
I'
I''
J
% of Wolbachia infected stalks
100
90
80
70
60
50
40
30
20
10
0
*
wMel
wMelCS
wMelPOP

Figure 3.12: *Wolbachia* are homogenously distributed in somatic follicle cell precursors but enrich in PCs upon differentiation.

(A) Schematic of a *Drosophila* ovariole. (B) Schematic of gemarium, stage 3, and stage 4 egg chamber. PC/SC precursors are marked in yellow, matured PCs marked in red and lateral FCs marked in grey. *upon maturation, lateral FCs lose FasIII expression whereas PCs maintain a higher expression of FasIII. (C-C') A germarium, stage 3 and stage 4 egg chambers stained for *Wolbachia* (*w*MelPop, green), FasIII (red), and DNA (blue). (D-D''') is a higher magnification of the germarium showing no preferential *Wolbachia* accumulation between regions 2b and 3 where the PC/SC precursors reside (yellow dashed line in D''). (E-E''') is a stage 3 egg chamber with clearly specified posterior PCs (marked by strong FasIII staining). (E") *Wolbachia* clearly accumulate in the posterior PCs (red dashed lines) whereas the anterior PC/SC precursors (yellow dashed lines) have no preferential *Wolbachia* accumulation. (F-F") Stage 4 egg chamber with high *Wolbachia* accumulation in the matured polar cell. (G-G'') Germarium stained for DNA (blue), *Wolbachia* (green), and Eya (red). PC/FC precursors can be identified by their lack of Eya staining (yellow dashed line in G''). (G') We observe no higher accumulation of *Wolbachia* in the PC/FC precursors (yellow dashed lines) as compared to lateral follicle cells (green dashed lines). (H) In all 3 *Wolbachia* strains, there is a similar proportion of infected PC/SC precursor cells as lateral follicle cells. Error bars show 95% c.i. (I) Density quantification shows that in all 3 *Wolbachia* strains tested, *Wolbachia* density in the PC/SC precursors (yellow dashed lines in D" and E") is similar to the lateral follicle cells (green dashed lines in D"' and E"'). (J) Quantification shows that *Wolbachia* density in cells

lacking Eya staining (yellow dashed lines in G') is comparable to cells marked with high Eya (green dashed lines in G'). *Wolbachia* labeled by 16SrRNA FISH. N≥18 for each genotype. Scale bars = 10μm. Error bars show s.e.m. Student's t test.

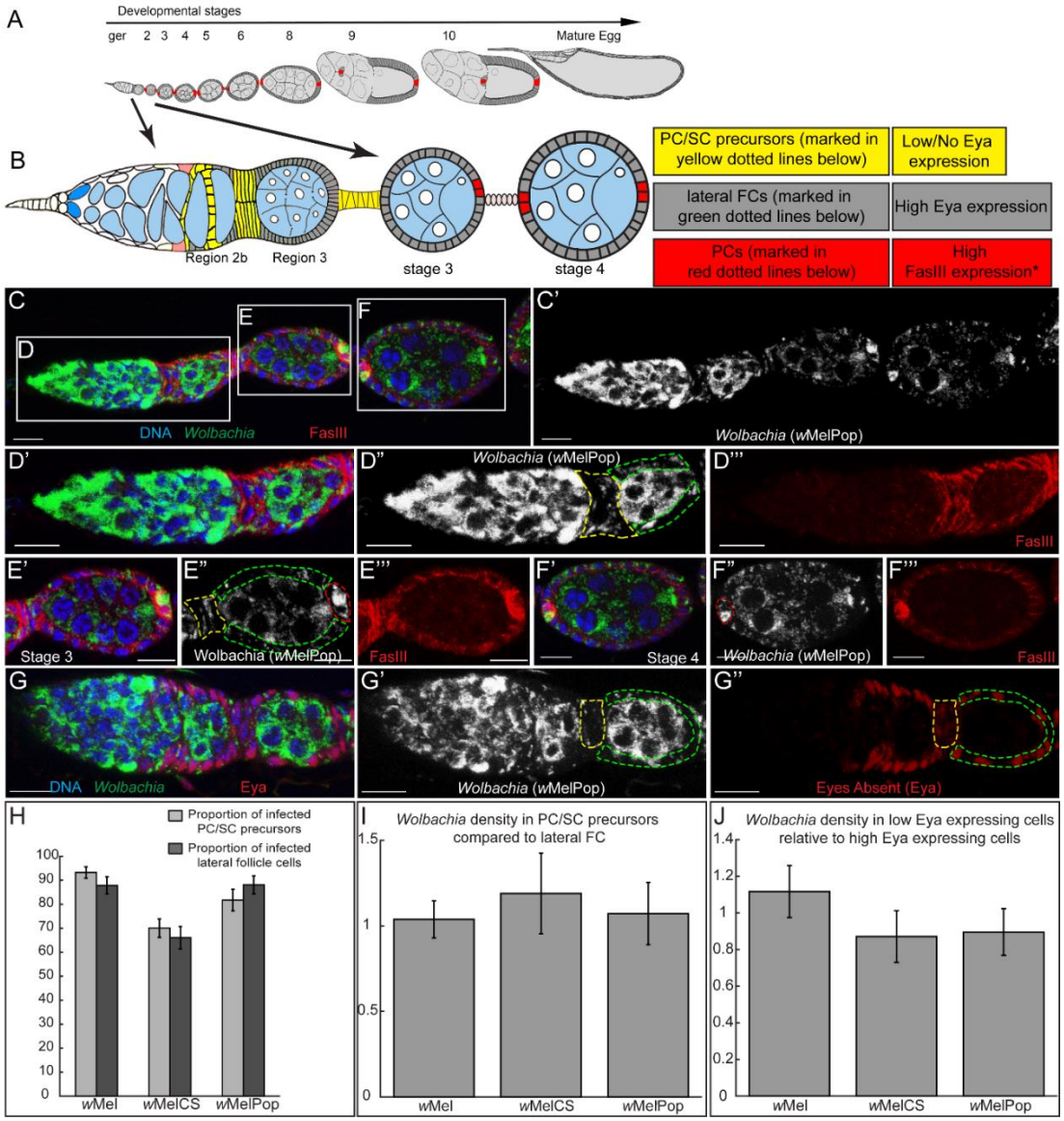


Figure 3.13: *Wolbachia* accumulate in ectopic PCs by over-replication.

(A) In GR1>eyaRNAi flies, *Eyes absent (Eya)* RNAi is present from stage 3 egg chamber onwards (black arrow on top). Knock down of *Eya* (a PC fate inhibitor), induces transformation of lateral FCs into ectopic PCs (red cells pointed by red arrow in diagram), with some unaffected PCs (green arrow). (B, C) ectopic PCs (labelled with FasIII, red) show substantial *Wolbachia* (*w*MelCS, green) accumulation (A'-A'', stage 5; B'-B", stage 8). (D) *Wolbachia* density in the ectopic PCs is equivalent to the density in the normal PCs for each corresponding stage in the same ovaries. Even though ectopic PCs volumes range from 6–fold (stage 8) to 13–fold (stage 5) higher than the corresponding normal PCs (E), the amount of *Wolbachia* in the surrounding FsC is the same. This indicates that there is no uptake of *Wolbachia* from lateral FCs to ectopic PCs, suggesting that replication of *Wolbachia* in the PCs is the mechanism of PC tropism. *Wolbachia* labeled by 16SrRNA FISH. N≥19 for each stage and genotype. Scale bars = 10um. Error bars show s.e.m., ns $p>0.05$, ***$p<0.001$, Student's t test.

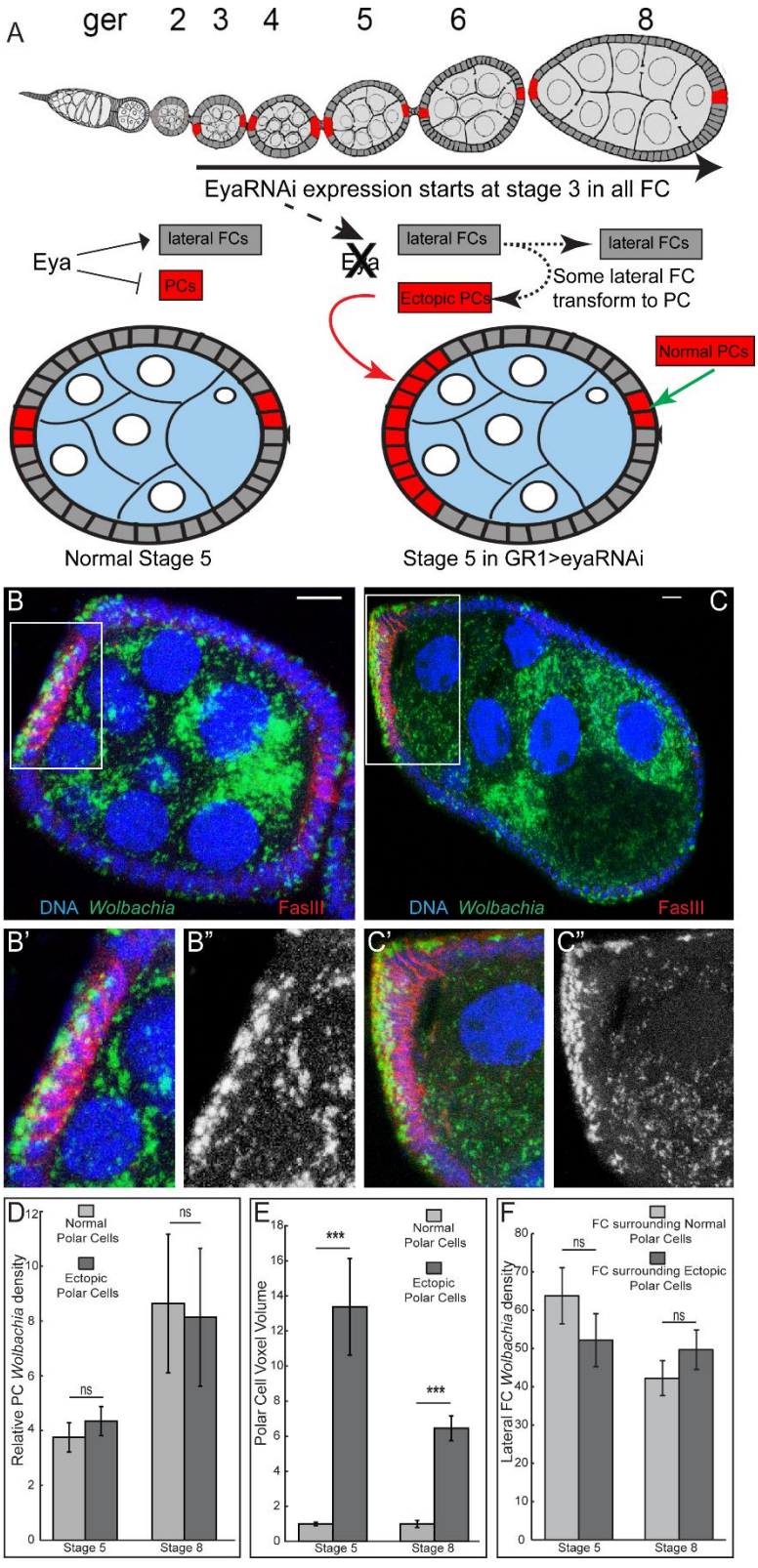
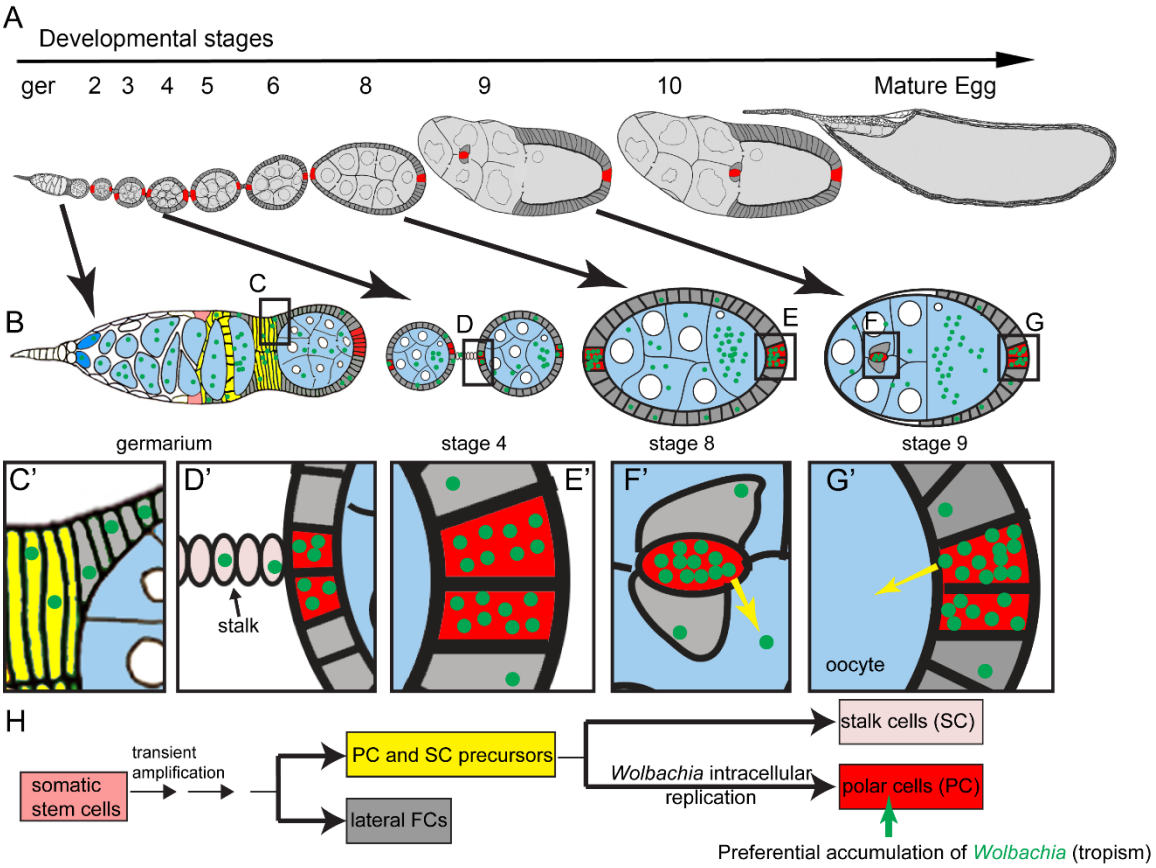

A
ger
2
3
4
5
6
8
EyaRNAi expression starts at stage 3 in all FC
Eya
lateral FCs
PCs
lateral FCs
lateral FCs
Some lateral FC
transform to PC
Ectopic PCs
Normal PCs
Normal Stage 5
Stage 5 in GR1>eyaRNAi
B
C
DNA Wolbachia FasIII
DNA Wolbachia FasIII
B'
B"
C'
C"
D
Normal
Polar Cells
Ectopic
Polar Cells
ns
ns
Relative PC Wolbachia density
Stage 5
Stage 8
E
Normal
Polar Cells
Ectopic
Polar Cells
***
***
Polar Cell Voxel Volume
Stage 5
Stage 8
F
FC surrounding Normal
Polar Cells
FC surrounding Ectopic
Polar Cells
ns
ns
Lateral FC Wolbachia density
Stage 5
Stage 8

Figure 3.14: Model for developmental accumulation of *Wolbachia* during PC morphogenesis.

(A) Schematic of *Drosophila* egg maturation with PCs marked in Red. (B) Diagram of various representative stages in polar cell (PC) development with *Wolbachia* (green circles) accumulation increasing with PC development. (C) Magnification of germarium showing the common polar cell (PC) and stalk cell (SC) precursor (marked in yellow) without a preferential accumulation of *Wolbachia*. (D-D') Magnification of a stage 4 PCs showing the beginning of preferential *Wolbachia* accumulation specifically in the PCs (red). The stalk cells (pink) do not have a high *Wolbachia* accumulation. (E-E') Magnification of stage 8 PCs showing a large accumulation of *Wolbachia* specifically in the PCs as compared to the lateral follicle cells (FC). (F-F') Magnification of a migrating anterior PCs in a stage 9 egg chamber shows high *Wolbachia* accumulation and a potential route for *Wolbachia* into the germline (blue). (G-G') Magnification of a posterior PCs in a stage 9 egg chamber with high *Wolbachia* accumulation. (H) Model for PCs and SCs differentiation adapted from Tworoger *et al*. and Chang *et al*. (Tworoger *et al.* 1999, Chang *et al.* 2013) showing a common precursor for SC and PC which is different from lateral follicle cells (FCs). Our data suggest that *Wolbachia* preferentially replicate in the PC lineage after its differentiation (green arrow).


A
Developmental stages
ger
2
3
4
5
6
8
9
10
Mature Egg
B
C
D
E
F
G
germarium
stage 4
stage 8
stage 9
C'
D'
E'
F'
G'
stalk
oocyte
H
somatic stem cells
transient amplification
PC and SC precursors
lateral FCs
Wolbachia intracellular replication
stalk cells (SC)
polar cells (PC)
Preferential accumulation of Wolbachia (tropism)

CHAPTER 4 Canonical Wnt signaling modulates *Wolbachia* intracellular density in insects

4.1 Introduction

Tissue tropism is an essential aspect of microbial-host interactions. The tissues preferentially infected by a microbe can determine the consequences of infection for the host as well as the successful establishment and transmission for the microbe. *Wolbachia* are maternally transmitted intracellular bacteria that have tropism to the female germline in hosts. Even in cases where *Wolbachia* are horizontally transmitted, they must colonize the germline to ensure maternal transmission necessary for establishment in a population. Although *Wolbachia* have a strong selective pressure to infect the germline, infection of various somatic tissues is widespread (Hosokawa *et al.* 2010, Landmann *et al.* 2010). In fact, one of the first tissues infected by *Wolbachia* upon infection of a naive host is the somatic stem cell niche in the ovary (Frydman *et al.* 2006). Understanding host-*Wolbachia* interactions on a molecular level will allow us to exploit *Wolbachia*'s dependence on host pathways and modulate *Wolbachia* density using small molecule agonists.

Here we investigate the role of host Wnt signaling in *Wolbachia* density control. Wnt signaling is a conserved pathway involved in cell proliferation and patterning in all animals (Bejsovec 2013). Canonical Wnt signaling (Fig. 4.1E) is activated when the ligand Wnt binds to the transmembrane receptor Frizzled (Fz). Disheveled (Dsh) is then recruited to the cell membrane which subsequently inactivates the protein complex of Axin, Adenomatous Polyposis Coli (APC), and Shaggy/Glycogen Synthase Kinase 3β (Sgg/GSK3β), leading to inhibition of Armadillo/β-catenin (Arm) phosphorylation and

degradation. Unphosphorylated Arm translocates to the nucleus and activates gene expression (Bejsovec 2013, Stamos and Weis 2013) (Fig. 4.1E). Although a direct correlation between Wnt pathway and *Wolbachia* has never been shown, three major lines of evidence indicate a likely *Wolbachia*/Wnt interaction:

(1) In the *Drosophila* gonads, *Wolbachia* have tropism to tissues with active Wnt signaling. For instance, *Wolbachia* preferentially replicate in the female germline and somatic stem cell niches (Fig. 4.1C,C') (Frydman *et al.* 2006, Toomey *et al.* 2013) where Armadillo (Arm) mediated Wnt signaling regulates somatic stem cell maintenance (Song and Xie 2003) and the DE-cadherin mediated cell adhesion role of Arm is essential for anchoring both the germline and somatic stem cells to their respective niches (Song and Xie 2002, Song *et al.* 2002, Song and Xie 2003). *Wolbachia* tropism is also reported in the polar cells (Fig. 4.1D,D'), a specialized subset of epithelial cells essential for proper morphogenesis (Kamath *et al.* 2018) where Wnt signaling is important for polar cell specification (Dai *et al.* 2017). In the testis, *Wolbachia* infect the stem cell niche, the hub (Fig. 4.1A) (Toomey and Frydman 2014) where DE-cadherin/Armadillo mediated adhesion has been well characterized (Voog *et al.* 2008) and more recently the involvement of Wnt signaling in hub and stem cell development in the testis has been described (Deshpande *et al.* 2016). The correlative tropism of *Wolbachia* to Wnt-defined cell types suggests *Wolbachia* may also utilize this pathway.

(2) Many intracellular bacteria have been shown to interact with Wnt signaling. Wnt signaling mediated innate immunity has been demonstrated in phagocytosis of microorganisms (Maiti *et al.* 2012, Zhu and Zhang 2013). *Salmonella*, a gastrointestinal

pathogen, inhibits the degradation of Armadillo leading to activation of Wnt signaling in epithelial cells (Sun *et al.* 2004, Liu *et al.* 2010). More recently, *Mycobacterium tuberculosis* has been shown to modulate Wnt signaling in macrophages to promotes its intracellular survival (Villasenor *et al.* 2017). *Ehrlichia chaffeensis*, an intracellular human pathogen very closely related to *Wolbachia*, suppresses Armadillo phosphorylation upon infection to promote Wnt signaling (Luo *et al.* 2015). These findings suggest that *Wolbachia* may also interact with or regulate Wnt signaling in its host.

(3) *Wolbachia* have also been shown to affect glycogen biosynthesis (de Abreu *et al.* 2014, Dobson *et al.* 2015), a reaction that is modulated by GSK3, a major negative regulator of Wnt signaling. For instance, *Wolbachia* infected *Drosophila* have significantly elevated glycogen levels (Dobson *et al.* 2015), whereas knockdown of GSK3 in *Aedes fluviatilis* led to a reduction of *Wolbachia* levels by reducing host glycogen levels (da Rocha Fernandes *et al.* 2014). Moreover, the depletion of *Wolbachia* led a reduction of GSK3 expression in *Brugia malayi* and *Litomosoides sigmodontis*, a rodent filarial nematode (Voronin *et al.* 2016). Taken together, these observations provide a strong precedence for the role of Wnt pathway in intracellular bacterial survival including *Wolbachia* in the *Drosophila* gonads.

Wolbachia are a novel tool to control the spread of devastating vector-transmitted diseases including Malaria, Dengue, Zika, Chikungunya, Yellow fever, and West Nile Virus. When *Wolbachia* from *Drosophila melanogaster* (*w*Mel) are introduced into a mosquito host, they reduce the establishment as well as transmission of many pathogens in the mosquito including Dengue and Zika virus (Kambris *et al.* 2009, Moreira *et al.* 2009,

Bian *et al.* 2010, Walker *et al.* 2011, Blagrove *et al.* 2012, Bian *et al.* 2013, Schultz *et al.* 2017). Studies suggest that there is a correlation between *Wolbachia* densities and virus interference in hosts (Osborne *et al.* 2009, Frentiu *et al.* 2010, Lu *et al.* 2012, Osborne *et al.* 2012, Chrostek *et al.* 2013). Nonetheless, we have limited understanding of the mechanisms that control *Wolbachia* intracellular levels.

In this study, using transgenic flies we show that upregulation of Wnt signaling results in *Wolbachia* intracellular accumulation in both somatic and germline cells of the *Drosophila* gonads. Conversely, inhibition of signaling, reduces *Wolbachia* intracellular levels. Moreover, we use small molecule Wnt agonists to significantly increase *Wolbachia* levels in vectors of public health relevance including *Aedes aegypti* and *Aedes albopictus*. This study provides a novel approach for control of *Wolbachia* intracellular density in vectors with the aim of disease control.

4.2 Armadillo knockdown reduces *Wolbachia* density in the testis' hub

To investigate whether Arm plays a role in *Wolbachia* intracellular accumulation, we knocked down Arm by expressing armRNAi in the hub under the control of *upd*-Gal4. In this experiment, we used two strains of *Wolbachia* (*w*Mel and *w*MelCS) which accumulate at different densities in the hub (Toomey *et al.* 2013). Upon knockdown of *arm*, we observed a reduction in *Wolbachia* accumulation in the hub (Fig. 4.2A,B). Relative *Wolbachia* density in the hub was quantified by normalizing *Wolbachia* density in the hub to the *Wolbachia* density in the surrounding region (see chapter 2). Upon quantification, we calculated about a four-fold reduction in *w*Mel density and about a three-fold reduction *w*MelCS density in the hub (Fig. 4.2C). Knockdown of Arm in the hub was

assessed by immunohistochemistry (Fig. 4.3A,B). These observations indicate that knockdown of *arm* decreases *Wolbachia* densities in the hub.

4.3 Constitutively active Wnt signaling increases *Wolbachia* density in the hub

Arm has two roles in the cell. The first is as an important constituent of the cell-cell adherens junctions. The second role is in the Wnt signaling pathway. To assess if *Wolbachia* intracellular density is altered by Arm via Wnt signaling, we expressed a constitutively active Armadillo named Arm^{S10}. Arm^{S10} is a transgenic protein which has a 54 amino acid deletion in the N-terminal domain and as a result is missing a GSK/Zw3 phosphorylation site, as well as a ubiquitination site (Pai *et al.* 1997). Because of this deletion, Arm^{S10} is resistant to degradation by its destruction complex, allowing for accumulation of cytosolic Arm. This results in the activation of the Wnt pathway in the absence of the Wnt signal. We expressed Arm^{S10} in the hubs under the control of the *upd*-Gal4 driver. We temporally controlled expression of Arm^{S10} using the $Gal80^{ts}$ system since constitutive expression of this construct is pupal lethal. At the restrictive temperature of 18ºC, flies were allowed to develop until adulthood. One-day old flies were shifted to the permissive temperature of 29ºC and aged for seven days. We observed that *Wolbachia* levels were highly elevated in the hubs which were expressing Arm^{S10} (Fig. 4.2 D,E). Quantification showed that *w*Mel density was increased by about 3.5-fold and *w*MelCS density was increased by about 2.5-fold in the hub (Fig. 4.2F).

Moreover, we found that Wnt signaling affects *Wolbachia* density in the hub early in hub development. Hubs are specified during embryogenesis and develop throughout the larval stages (Le Bras and Van Doren 2006). As the expression of Arm^{S10} was pupal lethal,

we assessed *Wolbachia* levels in L3 larval hubs expressing Arm^{S10}. We observed that *Wolbachia* levels in larval hubs were elevated upon expression of Arm^{S10} (Fig. 4.5A,B). Quantification showed that *w*Mel density is elevated about two-fold and *w*MelCS density is elevated about three-fold (Fig. 4.5C) in larval hubs upon expression of Arm^{S10}. These observations support our hypothesis that Arm acts via the Wnt pathway to affect intracellular *Wolbachia* density in the hubs of *Drosophila* testes.

4.4 Armadillo does not affect *Wolbachia* levels via adherens junction

In the hub, Arm along with *Drosophila* E-Cadherin anchors the stem cells to adjacent cells via adherens junctions (Leatherman and Dinardo 2010). Adherens junctions including E-cadherin and Arm have been shown to be important for bacterial entry into host cells (Mengaud *et al.* 1996). To investigate if Arm-dependent adherens junctions facilitate high intracellular *Wolbachia* levels in the hub, we knocked down E-Cadherin, an essential partner of Arm in the adherens junctions rendering the role of Arm in adherens junctions non-functional. Interestingly, we observed no change in *Wolbachia* levels upon expression of DE-Cad RNAi in the hubs (Fig. 4.4). We therefore conclude that Arm's role in the adherens junctions is not necessary and rather Arm's role in the Wnt signaling pathway is likely to play a role in *Wolbachia* accumulation.

4.5 Downregulation of Wnt signaling by Disheveled knockdown decreases *Wolbachia* level

To further investigate the role of canonical Wnt signaling in *Wolbachia* intracellular accumulation, we knocked down additional genes in the pathway (see Fig. 4.1E). We first downregulated Disheveled (Dsh), a positive regulator of Wnt pathway by expressing RNAi

in the hub under the control of *upd*-Gal4 (Bejsovec 2013). After activation by the receptor Fz, Dsh inactivates the Arm destruction complex thereby promoting cytosolic accumulation of Arm and Wnt signaling (Fig. 4.1C). Knockdown of *dsh* in hubs led to a reduction in *Wolbachia* levels (Fig. 4.6A,B). *w*Mel density was reduced by two-fold and *w*MelCS density was reduced by six-fold (Fig. 4.6C). This shows that knockdown of Wnt pathway leads to a reduction in *Wolbachia* levels intracellularly.

4.6 Upregulation of Wnt signaling by GSK3β knockdown increases *Wolbachia* levels

Additionally, to further confirm the role of canonical Wnt signaling in *Wolbachia* accumulation, we knocked down Shaggy/GSK3β (Sgg), a negative regulator of the pathway. Sgg is a kinase and part of the Arm destruction complex (Stamos and Weis 2013). Sgg phosphorylates Arm marking Arm for ubiquitination and proteasome mediated degradation (Stamos and Weis 2013). Knockdown of Sgg is sufficient to upregulate Wnt signaling. Upon knockdown of *sgg*, we observed a 1.5-fold increase in *Wolbachia w*Mel density in the hub (Fig. 4.6D-F). Consistently, *w*MelCS density was increased four-fold (Fig. 4.6F). In conclusion, upregulation of Wnt signaling increases *Wolbachia* levels and downregulation of Wnt signaling decreases *Wolbachia* levels in the male hub.

4.7 Wnt signaling affects *Wolbachia* density in the polar cells of the ovary

To ascertain if the effect of Wnt signaling on *Wolbachia* accumulation is conserved across cell types in the female *Drosophila*, we investigated the polar cells (PCs). PCs are an ovarian somatic cell type which are infected with *Wolbachia* at a high density (Kamath *et al.* 2018). Arm has been shown to be highly upregulated in these cells by immunostaining (Peifer *et al.* 1993). Moreover, recently the Wnt pathway has been shown to assist in PC

specification (Dai *et al.* 2017). Considering our previous results, we hypothesized that Wnt signaling via Arm in PCs would affect intracellular *Wolbachia* density. We either knocked down Wnt signaling by expressing armRNAi or upregulated Wnt signaling by expressing Arm^{S10} specifically in the PCs under the control of *upd*-Gal4. To quantitatively assess changes in *Wolbachia* density upon modulation of Wnt signaling, we normalized *Wolbachia* density in the PC to *Wolbachia* density in the surrounding follicle cells (FCs). We compared relative *Wolbachia* density in the PC in stage 8 egg chambers, the middle stage of oogenesis.

Knockdown of Arm using armRNAi led to a reduction in intracellular *Wolbachia* density in the PCs (Fig. 4.7A,B). Quantification showed about a two-fold reduction of both *w*Mel and *w*MelCS infected flies (Fig. 4.7C). Conversely, upregulation of Wnt signaling by Arm^{S10} expression led to a statistically significant increase in intracellular *Wolbachia* density (Fig. 4.7D,E). Quantification showed about a 1.5-fold increase of *w*Mel density and a 1.7-fold increase of *w*MelCS density in PCs (Fig. 4.7F). Together, these observations suggest that Wnt signaling plays a role in intracellular *Wolbachia* density in the PCs. Modulation of Wnt signaling is effective to change intracellular *Wolbachia* levels in the PCs.

4.8 Upregulation of Wnt signaling is sufficient to drive *Wolbachia* tropism

We have demonstrated that activation of Wnt signaling is sufficient to increase *Wolbachia* density in various insect tissues. However, because *Wolbachia* have tropism to these tissues, this does not show that Wnt signaling can act alone to promote *Wolbachia* density. To address this, we determined if activating Wnt signaling can induce *Wolbachia*

tropism to normally uninfected tissues. We chose the germline stem cell niche (GSCN) of the *Drosophila* ovary to test this hypothesis because only certain strains of *Wolbachia* have tropism to the GSCN (Toomey *et al.* 2013). In *D. mel*, *w*Mel has almost no tropism to the GSCN whereas *w*MelCS has a low amount of tropism to these cell types (Toomey *et al.* 2013). We overexpressed Wnt signaling in the GSCN by expressing Arm^{S10} under the control of *bab*-Gal4 (Sahut-Barnola *et al.* 1995, Cabrera *et al.* 2002). In control flies, most ovarioles showed very little to no *Wolbachia* tropism to the GSCN (Fig. 4.8A). However, activation of Wnt signaling by Arm^{S10} expression led a marked increase in ovarioles which had a high *Wolbachia* tropism to the GSCN (Fig. 4.8B). We quantified this phenotype by calculating the percentage of GSCN with *Wolbachia* tropism (% of GSCN which looked like Fig. 4.8B). In control *w*Mel infected flies, only about 8% of GSCN had *Wolbachia* tropism whereas upon expressing Arm^{S10}, we found about 26% GSCN had *Wolbachia* tropism (Fig. 4.8C). Similarly, *w*MelCS GSCN tropism increased from 16.6% in control flies to about 52.5% in Arm^{S10} expressing flies (Fig. 4.8C). Next, we calculated *Wolbachia* density in the GSCN which have tropism by fluorescence quantification. Quantification was performed only in GSCN which had tropism (like Fig. 4.8B). We observed a six-fold increase in *w*Mel levels and a 3.6-fold increase in *w*MelCS levels in the GSCN upon expression of Arm^{S10} (Fig. 4.8D). These observations show that we can drive *Wolbachia* tropism to uninfected tissues by over-activating Wnt signaling.

4.9 Wnt signaling affects *Wolbachia* density in female germline

Elevated *Wolbachia* levels in the female germline are important for maternal transmission to the next generation. Our results until now suggest that modulating Wnt

signaling is sufficient to regulate *Wolbachia* intracellular levels in somatic cell types of *Drosophila* gonads. We next wanted to determine whether Wnt signaling is involved in modulating *Wolbachia* levels in the female germline. To test this, we knocked down Wnt signaling by expressing *arm*RNAi and in an independent experiment upregulated Wnt signaling by expressing Arm^{S10} under the control of *nanos*-Gal4 (Rorth 1998). A *Drosophila* egg chamber is composed of 16 germ cells, including one oocyte and 15 nurse cells, and a thin layer of somatic follicle cells. As the germline cells are highly polyploid and occupy a large volume of the egg chamber, they represent a substantial proportion of the genetic material in an ovary (Fig. 4.1B). Therefore, we estimate that DNA analysis of the whole ovary is representative of the germline. We quantified changes in germline *Wolbachia* level by real time qPCR of whole ovarian DNA (Fig. 4.9A).

Knockdown of Wnt signaling by *arm*RNAi decreased *Wolbachia* levels in the germline by ~30% for both *w*Mel and *w*MelCS strains (Fig. 4.9B). Conversely, upregulation of Wnt signaling by Arm^{S10} caused ~60% increase in *w*Mel levels and ~40% increase in *w*MelCS levels in the germline (Fig. 4.9C). Taken together, these results suggest a role for Wnt signaling in modulating *Wolbachia* levels and potentially affecting maternal transmission in the female germline.

4.10 Chemical induction of Wnt signaling is sufficient to enhance *Wolbachia* levels in cell lines and whole insects

Increasing whole insect *Wolbachia* density has been shown to promote *Wolbachia*'s anti-pathogen phenotype enhancing the control of vector disease transmission (Joubert *et al.* 2016). We hypothesized that activating Wnt signaling in *Wolbachia* infected

insects may increase total *Wolbachia* levels in the insect. Wnt signaling can be activated by small molecule agonists to enhance *Wolbachia* levels. Lithium ions (Li^+) are a known inhibitor of Shaggy (Sgg/GSK3β), an inhibitor of the Wnt pathway (Stambolic *et al.* 1996, Chalecka-Franaszek and Chuang 1999, Ryves and Harwood 2001).

We treated *Drosophila* cell lines infected with *w*Mel (JW18), *Aedes aegypti* cell lines infected with *w*AlbB (Aag2) and *Aedes albopictus* cell lines infected with *w*AlbB (Aa23) with Li^+ and measured *Wolbachia* levels by qPCR (Fig. 4.9A). Li^+ deactivation of Sgg by phosphorylation was confirmed by western blotting (Fig. 4.10). Li^+ treatment caused a 1.75-fold increase of *Wolbachia w*Mel in *Drosophila* cells (JW18). We also observed a 2.28-fold increase of *w*AlbB in *A. aegypti* mosquito (Aag2) cells and a 3.16-fold increase in *A. albopictus* mosquito (Aa23) cells (Fig. 4.9D). To assess the response of *Wolbachia* to systemic induction of Wnt signaling, we fed adult *w*Mel infected *D. melanogaster* with Li^+ and quantified *Wolbachia* levels in the whole flies. Li^+ treatment caused a 1.5-fold increase in *Wolbachia* levels in whole flies as assessed by qPCR (Fig. 4.9E). These results show that both in mosquitoes and *Drosophila*, *Wolbachia* levels can be modulated by affecting the Wnt pathway. Moreover, we can increase *Wolbachia* levels in whole insects by feeding them small molecule agonists of the Wnt pathway.

4.11 Discussion

Wolbachia, maternally transmitted obligate endosymbionts, colonize specific host tissues to facilitate their efficient transmission. There is a growing body of literature suggesting a role for Wnt signaling pathway in bacterial entry and intracellular survival (Sun *et al.* 2004, Liu *et al.* 2010, Luo *et al.* 2015, Villasenor *et al.* 2017). Here we show

for the first time, a role for canonical Wnt signaling in *Wolbachia* stem cell niche tropism and intracellular growth. These findings have implications in understanding basic biology of endosymbionts as well as in vector disease control as *Wolbachia* reduce vector competency of mosquitoes (Moreira *et al.* 2009, Bian *et al.* 2010, Walker *et al.* 2011, Blagrove *et al.* 2012, Osborne *et al.* 2012).

Previous studies have shown *Wolbachia* tropism to specific cells of *Drosophila* gonads including the female germline, somatic stem cell niche (SSCN) (Frydman *et al.* 2006), germline stem cell niche (GSCN) (Toomey *et al.* 2013), polar cells (PCs) (Kamath *et al.* 2018) in ovaries, and the hub (Toomey and Frydman 2014) in testes. The mechanism for *Wolbachia* tropism to these specific cell types are unknown. Armadillo (Arm) mediated Wnt signaling is implicated in the development of all these cell types (Song and Xie 2002, Song and Xie 2003, Deshpande *et al.* 2016, Dai *et al.* 2017). These reports along with studies implicating Wnt signaling in intracellular bacterial survival (Sun *et al.* 2004, Liu *et al.* 2010, Luo *et al.* 2015, Villasenor *et al.* 2017) led us to investigate whether *Wolbachia* rely on host Wnt signaling to colonize and survive in specific host cells. Using the hub, a molecularly well characterized cell type, we downregulated Wnt signaling by knocking down Arm or Disheveled (Dsh), positive regulators of the canonical Wnt pathway (Bejsovec 2013). Upon downregulation, we observed a decrease in intracellular *Wolbachia* levels. Conversely, upon upregulation of Wnt pathway by knockdown of Shaggy (Sgg), a canonical Wnt signaling inhibitor (Bejsovec 2013, Stamos and Weis 2013), we observed an increase in *Wolbachia* density. By increasing Wnt signaling by expression of Arm^{S10}, a constitutively active Arm (Pai *et al.* 1997), we observed an increase of *Wolbachia*

infection. We recapitulated these findings in PCs as well as in the female germline, a tissue relevant for maternal transmission. Taken together, these results suggest a role for canonical Wnt signaling in *Wolbachia* intracellular accumulation.

The results summarized above show upregulation of Wnt signaling as a mechanism for *Wolbachia* tropism. Fig. 4.11 describes our model of how Wnt signaling drives *Wolbachia* tropism. In cell types such as the hub, PCs and female germline, the Wnt ligand binds the receptors leading to activation of a cascade that ends up dissociating Armadillo from the destruction complex. This activates canonical Wnt signaling leading to some downstream effects possibly including blockage of autophagy. These processes in turn stimulate high *Wolbachia* intracellular growth in these cell types. In other cells, the lack of the Wnt ligand leads Armadillo being targeted for proteasomal degradation. Here, there is no activation of Wnt signaling leading to a lack of *Wolbachia* intracellular growth and tropism. These mechanisms drive *Wolbachia* tropism to cell types with high Wnt signaling.

Our model predicts that upregulation of Wnt signaling will drive *Wolbachia* tropism. We have previously described that *Wolbachia* have variable tropism to GSCN as determined by *Wolbachia* strains. *w*Mel and *w*MelCS have low GSCN tropism in *Drosophila melanogaster* (Toomey *et al.* 2013). Although this tropism is determined by *Wolbachia* strains, we hypothesized that ectopic Wnt signaling could overcome this *Wolbachia* strain dependent effect and drive high frequency of GSCN tropism. This hypothesis was confirmed by driving *Wolbachia* tropism to the GSCN by ectopic Wnt signaling by Arm^{S10} expression. Therefore, we show that not only *Wolbachia* strain but also host factors can play a role in determining the patterns of stem cell niche tropism.

We have demonstrated the role of Wnt signaling in *Wolbachia* growth in two stem cell niches, the hub and the GSCN. Another stem cell niche displaying *Wolbachia* tropism is the SSCN. The ovarian SSCN has conserved, high *Wolbachia* tropism (Frydman *et al.* 2006, Toomey *et al.* 2013). Although there are no good molecular markers for the SSCN, the most posterior escort cell is generally considered to be the SSCN (Nystul and Spradling 2010, Sahai-Hernandez and Nystul 2013, Vlachos *et al.* 2015). The escort cells including the SSCN exhibit active Wnt signaling (Wang and Page-McCaw 2014, Wang and Page-McCaw 2018) which probably drives high *Wolbachia* titers in the SSCN. These observations further confirm that Wnt signaling plays a role in *Wolbachia* stem cell niche tropism.

In evolutionary terms, *Wolbachia* are under extreme selective pressure to colonize the female germline during horizontal and vertical transmission. *Wolbachia* interaction with Wnt signaling provides a selective advantage for both cases. In a newly infected insect, the SSCN is the first cell type to harbor high *Wolbachia* density and subsequently they invade the germline, facilitating horizontal transmission (Frydman *et al.* 2006). For vertical transmission, *Wolbachia* needs to grow at a high rate in the developing germline. Canonical Wnt pathway is also active in early germline development including the germline stem cells and developing cysts (Song and Xie 2003, Wang and Page-McCaw 2014, Upadhyay *et al.* 2018, Wang and Page-McCaw 2018). To successfully infect the germline at high levels, *Wolbachia* probably evolved mechanisms to grow in cells with high Wnt signaling activity. Here we show that upregulation of Wnt signaling in the germline also increases *Wolbachia* titers.

Both the hubs and the PCs have been shown to have very high levels of Arm (Peifer *et al.* 1993, Yamashita *et al.* 2003). Recent findings show that Wnt signaling is essential in early PC development (Dai *et al.* 2017) as well as hub development (Deshpande *et al.* 2016). *Wolbachia* tropism to hubs and PCs can be explained as a consequence of the selective pressure for *Wolbachia* to target the SSCN for horizontal transmission, and germline for vertical transmission.

Wolbachia's response to the host Wnt pathway adds to our understanding of how endosymbionts colonize specific host tissues. Some host processes known to affect *Wolbachia* levels are directly or indirectly regulated by Wnt signaling. These host processes include autophagy (Voronin *et al.* 2012), actin cytoskeleton (Newton *et al.* 2015) (Axelrod *et al.* 1998, Axelrod 2001), and insulin signaling pathways (Serbus *et al.* 2015) (Yoon *et al.* 2010, Palsgaard *et al.* 2012). It remains to be ascertained whether these processes act in conjunction or independently of the Wnt pathway to affect *Wolbachia* intracellular densities. Identification and targeting of Wnt pathway downstream genes specific to *Drosophila* will provide more insights into these questions. Insights from other intracellular bacteria will also help us define a clearer role of Wnt signaling in *Wolbachia* growth. *Ehrlichia chaffeensis*, a human pathogen closely related to *Wolbachia*, upregulates Wnt signaling in infected phagocytes. This inhibits the fusion of the phagosome with the lysosome leading to the intracellular survival of the bacteria (Luo *et al.* 2015). *Wolbachia*, like *Ehrlichia*, could take advantage of autophagy inhibition by the Wnt pathway in infected cells.

Wolbachia infection of vectors like mosquitoes can reduce transmission of deadly diseases like Dengue, Zika virus and plasmodium (Moreira *et al.* 2009, Bian *et al.* 2010, Hughes *et al.* 2011, Walker *et al.* 2011, Bian *et al.* 2013, Schultz *et al.* 2017). However, pathogen blockage is dependent on *Wolbachia* density (Osborne *et al.* 2009, Frentiu *et al.* 2010, Lu *et al.* 2012, Osborne *et al.* 2012, Chrostek *et al.* 2013, Schultz *et al.* 2017). For instance, *w*AlbB when introduced into *Aedes aegypti* achieves high density and can block transmission of Dengue. Whereas, *w*AlbB levels do not reach a high enough density to block Dengue transmission by *Aedes albopictus* (Lu *et al.* 2012). Moreover, *w*AlbB can infect *Anopheles stephensii*, vectors of malaria, only transiently (Bian *et al.* 2013). We hypothesize that the use of small molecule Wnt agonists upregulate Wnt signaling and would promote higher *Wolbachia* levels in these insect vectors. Using Li^+, a Wnt agonist (Stambolic *et al.* 1996), we increased *Wolbachia* levels in *Drosophila* (JW18), *Aedes aegypti* (Aag2) and *Aedes albopictus* (Aa23) cell lines. Moreover, whole insect Li^+ treatment was successful in increasing *Wolbachia* levels. Being implicated in many cancers, there exist multiple drugs to target the Wnt pathway (Meijer *et al.* 2004, Barker and Clevers 2006, Chen *et al.* 2010, Blagodatski *et al.* 2014). These provide us with multiple options of drugs to increase *Wolbachia* density in mosquitoes and to facilitate the introduction of novel *Wolbachia* strains in mosquito cell lines. Recently, additional *Wolbachia* strains have been shown to have significant arboviral suppression (Schultz *et al.* 2017). Alternately, using drugs inhibitory to Wnt pathway, we can reduce *Wolbachia* levels. This approach can be utilized in conjunction with antibiotics like doxycycline to target *Wolbachia* elimination from filarial nematodes as part of the A-WOL (Anti-

Wolbachia) consortium. Eliminating *Wolbachia* from filarial nematodes is a promising novel approach to the treatment and control of onchocerciasis and lymphatic filariasis (Taylor *et al.* 2010).

Here we show a mechanism of *Wolbachia* tissue tropism. Host Wnt signaling, an essential part of oogenesis, is essential for *Wolbachia*'s intracellular accumulation in specific cell types of the *Drosophila* gonads. Moreover, utilizing small molecule agonists we demonstrate the ability to manipulate *Wolbachia* levels in *Drosophila* and mosquito cell lines. The findings here provide novel molecular insights into *Wolbachia*-host interactions and their spread in nature. Furthermore, these findings also provide novel methods to manipulate *Wolbachia* in non-model insect hosts with the aim of further enhancing *Wolbachia* based methods of vector control of human pathogens including Dengue, Zika, Malaria and West Nile Virus.

Figure 4.1: Schematic of *Drosophila* gonads and Wnt signaling.

(A) Drawing of a male *Drosophila* with two testes. (A') At the apical tip of the testis lies the hub (in red) that anchors the germline stem cells (gray circle) and cyst stem cells (white crescents). (B) Drawing of a female *Drosophila* with two ovaries. Each ovary is made up of 14-16 ovarioles. Egg development proceeds from anterior (left) to posterior (right). At the anterior tip of the ovariole lies the germarium (C). (C') Schematic of the germarium. The germline stem cell niche (GSCN, green) anchor two germline stem cells (red) that give rise to the whole germline (pink). The somatic stem cell niche (SSCN, green) anchor the follicle stem cells (dark blue) which give rise to the follicle cells that encircle and support the developing germline cyst (light blue). (D-D') Schematic of a stage 8 egg chamber. The germline (pink) is encircled by a monolayer of follicle cells (gray). Polar cells (PCs, red) are two specialized cells at either pole that help in proper development of the follicle cells. (E) A schematic of Wnt signaling. In the absence of Wnt signaling, Armadillo is targeted for proteasome mediated destruction (left). Upon activation of Wnt signaling, Armadillo degradation by is blocked leading to nuclear translocation of Armadillo and transcription of downstream genes (right).

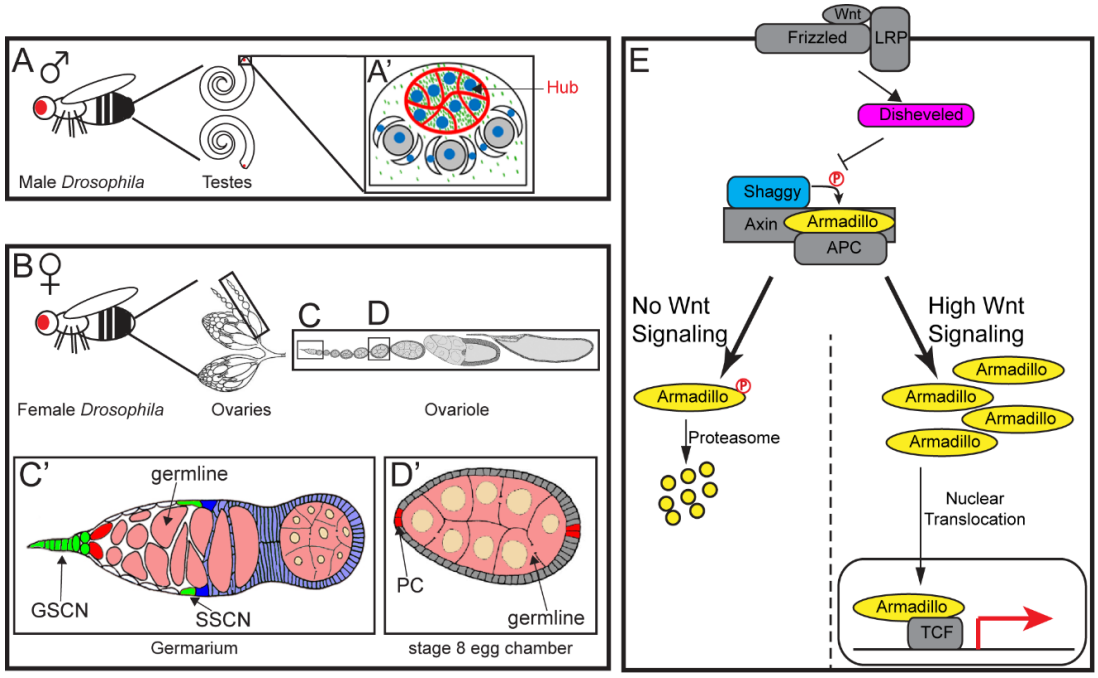

A ♂
Male Drosophila
Testes
A'
Hub
B ♀
Female Drosophila
Ovaries
C
D
Ovariole
C'
germline
GSCN
SSCN
Germarium
D'
PC
germline
stage 8 egg chamber
E
Wnt
Frizzled
LRP
Disheveled
Shaggy
Axin
Armadillo
APC
No Wnt Signaling
Armadillo
Proteasome
High Wnt Signaling
Armadillo
Armadillo
Armadillo
Armadillo
Nuclear Translocation
Armadillo
TCF

Figure 4.2 Modulation of Wnt signaling by controlling Armadillo levels affect *Wolbachia* density in the hub.

(A,B,D,E) Intracellular *Wolbachia* visualized in the hub by immunostaining against *Wolbachia* Hsp60 in green and hub marked by DE-cadherin staining (red). armRNAi expressing hubs (B,B') have a markedly reduced *Wolbachia* accumulation compared to controls (A,A'). Conversely, upon expression of Arm^{S10}, we observe a substantially increased *Wolbachia* load (E,E') compared to control hubs (D,D'). Quantifications (C,F) show that both observations lead to statistically significant change in *Wolbachia* levels for both *w*Mel and *w*MelCS strains of *Wolbachia*. Scale bars = 10µm. *p<0.05; **p<0.01; ***p<0.001, errorbars show SEM.

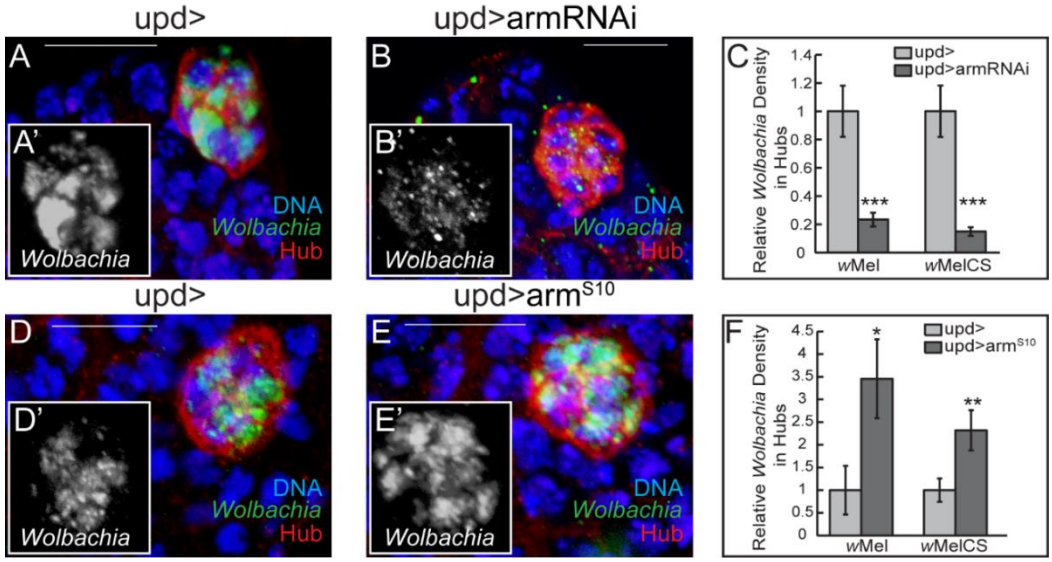


Figure 4.3: Efficient knockdown of Armadillo by RNAi.

(A-B) Armadillo levels visualized by staining for Armadillo in white, hub marked in red. Expressing *arm*RNAi in the hubs is sufficient to reduce the levels of Armadillo. (A',B') Insets make the observation clearer. (C-D) Armadillo staining of polar cells (marked in Red) shows that upon RNAi expression, Armadillo levels are significantly reduced in the polar cells (green arrowheads in the insets)

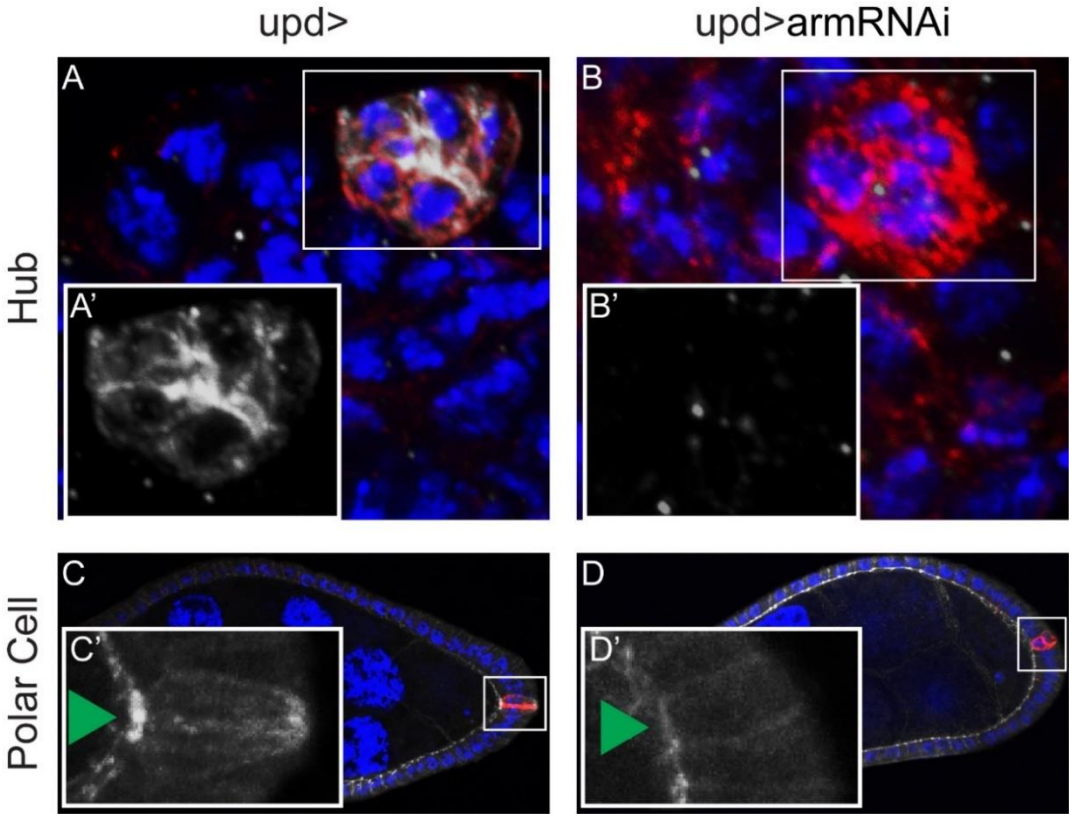


Figure 4.4: DE-cadherin knockdown has no effect on *Wolbachia* density in the hubs. (A) shows a control Hub stained for DE-cad in white and DN-cad in red to label the hub. (B) Expression of DE-cad RNAi is sufficient to knockdown DE-cad in the hubs. (') show DN-cad channels and (") show DE-cad channels. (C) Knockdown of DE-cadherin caused no effect on *Wolbachia* density in the hub. *Wolbachia* levels were quantified by image quantification as described in section 2.4.

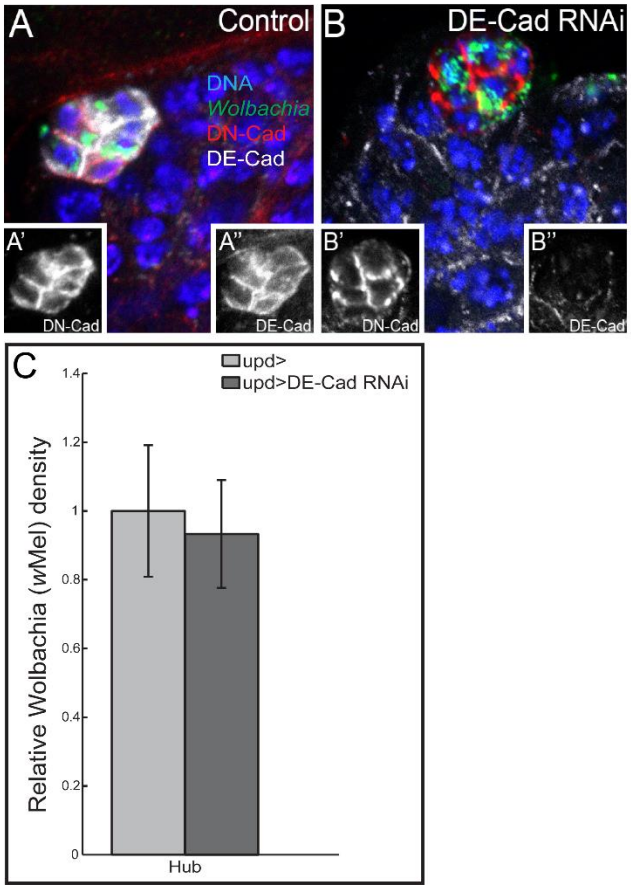


Figure 4.5: Upregulation of Wg/Wnt signaling is sufficient to drive high *Wolbachia* titers in larval hubs.

Intracellular *Wolbachia* visualized in the L3 larval hub by staining with a *Wolbachia* specific antibody in green and hub marked in red. Larval hub expressing Arm^{S10} (B) has an elevated *Wolbachia* level compared to control hub (A). Insets (A', B') show *Wolbachia* channel. Quantifications (C) show that both observations lead to statistically significant increase in *Wolbachia* levels for both *w*Mel and *w*MelCS strains of *Wolbachia*. **p<0.01; ***p<0.001, errorbars show SEM.

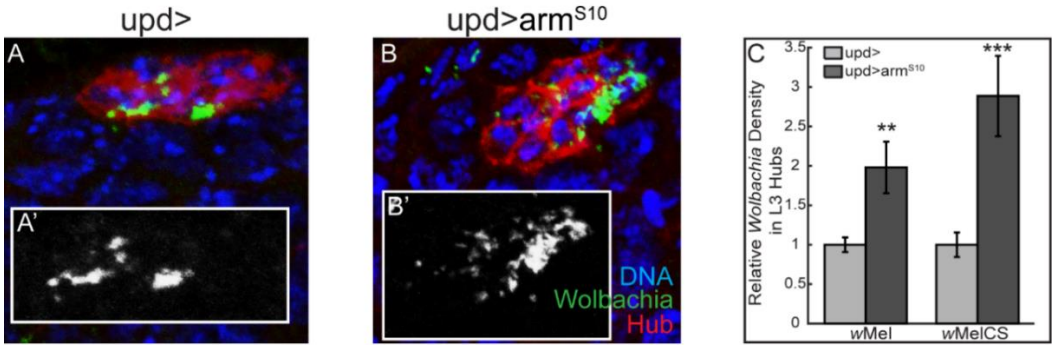


Figure 4.6: Modulation of Wnt signaling affect *Wolbachia* density in the hub.

(A,B,D,E) Intracellular *Wolbachia* visualized in the hub by staining for *Wolbachia* Hsp60 in green and E-cadherin to mark hubs in red. Knockdown of Dsh, a positive regulator of Wnt signaling, led to reduced *Wolbachia* accumulation (B,B') compared to control hubs (A,A'). Conversely, knockdown of GSK3β/Sgg, a negative regulator of Wnt signaling, leads to an increase in *Wolbachia* accumulation in the hub (E,E') compared to controls (D,D'). Quantifications (C, F) show that both manipulations lead to statistically significant changes in *Wolbachia* levels for both *w*Mel and *w*MelCS strains of *Wolbachia*. Scale bars = 10µm. *p<0.05; ***p<0.001, errorbars show SEM.

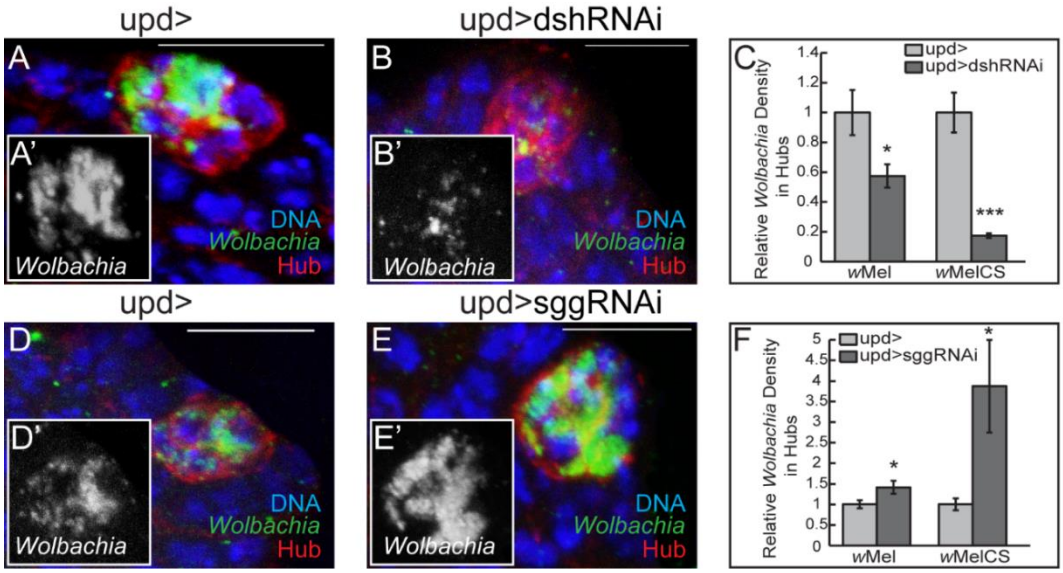


Figure 4.7: Modulation of Wnt signaling affects *Wolbachia* density in the polar cells. (A,B,D,E) Stage 8 egg chamber with intracellular *Wolbachia* visualized in the polar cells by FISH against *Wolbachia* 16srRNA (green) and PCs marked by immunostaining against a PC marker, FasIII (red). Knockdown of Wnt signaling by expressing armRNAi leads to a reduction of *Wolbachia* in the polar cells (B-B'') compared to the controls (A-A''). On the converse, upregulation of Wnt signaling by expressing Arm^{S10} leads to an increase in *Wolbachia* in the polar cells (E-E'') compared to the control (D-D''). White dashed lines in the insets mark the polar cell borders. Quantification of stage 8 polar cells (C, F) shows that both manipulations lead to statistically significant changes in levels for either *w*Mel and *w*MelCS strains of *Wolbachia*. Scale bars = 10µm. *p<0.05, errorbars show SEM.

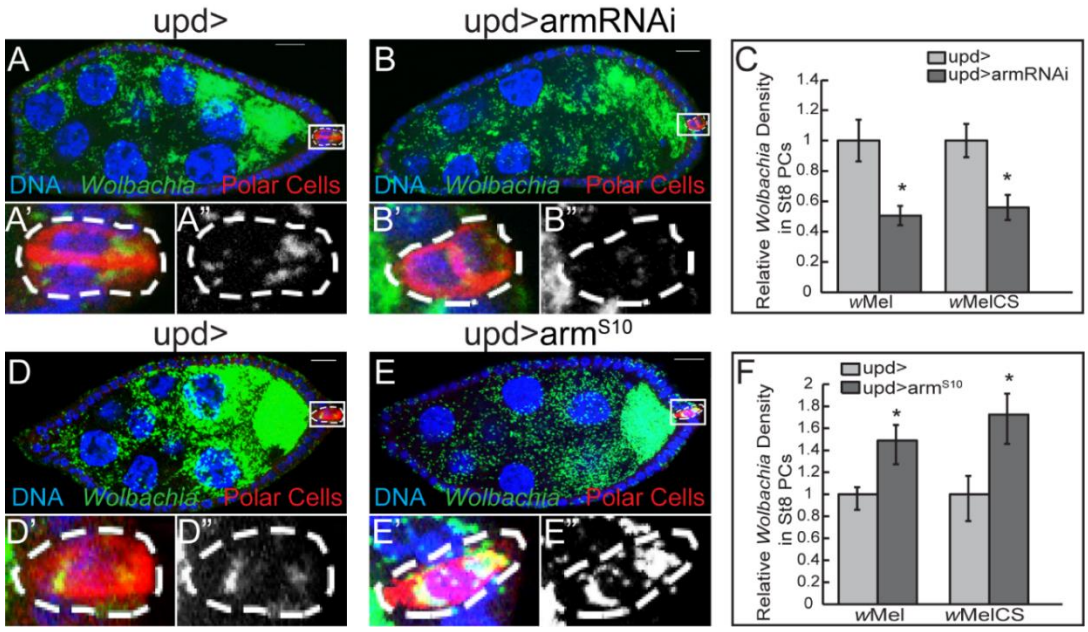


Figure 4.8: Upregulation of Wnt signaling is sufficient to drive *Wolbachia* tropism to the female germline stem cell niche (GSCN).

(A,B) *Wolbachia* are visualized by FISH against *Wolbachia* 16srRNA (green) and GSCN marked with immunostaining against Lamin C (red). Expression of Arm^{S10} in the GSCN (under the control of *bab*-gal4) leads to an increase in Wnt signaling and is sufficient to drive *Wolbachia* tropism to the GSCN (B,B'). Control GSCN (A,A') has no *Wolbachia* tropism. (C) Quantification of number of GSCN infected with *Wolbachia* shows that both *w*Mel and *w*MelCS strains have increased tropism to GSCN upon expression of Arm^{S10}. (D) Density calculations show that there is a higher *Wolbachia* density in GSCN upon Arm^{S10} expression for both *w*Mel and *w*MelCS strains. Scale bars = 10µm. *p<0.05, errorbars show SEM.

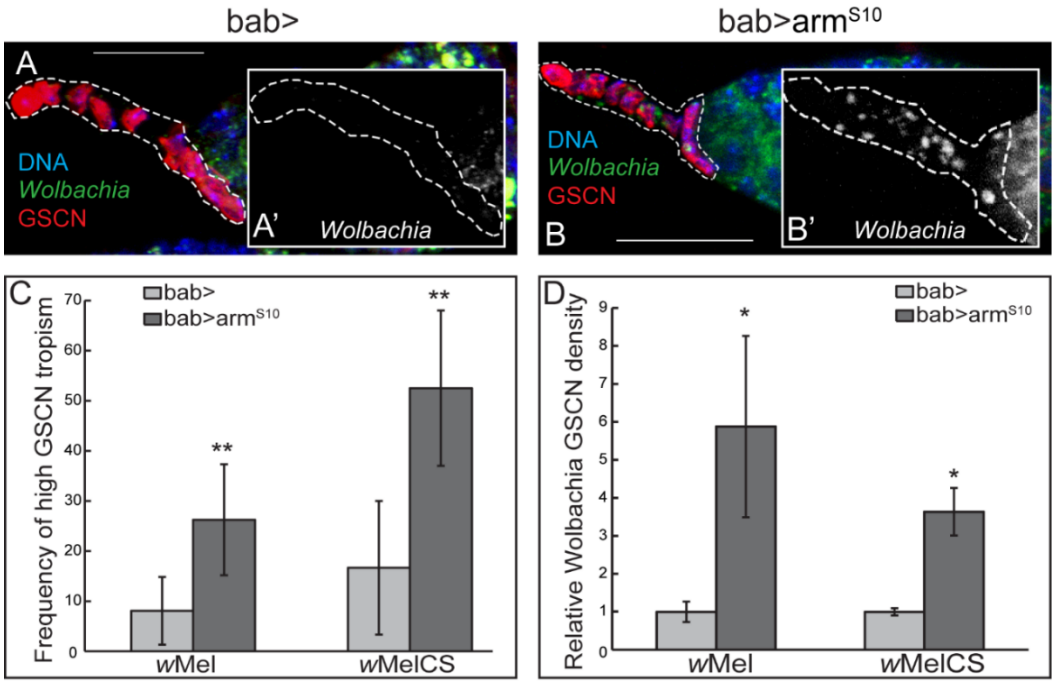


Figure 4.9: Modulation of Wnt signaling affects *Wolbachia* in the female germline, cell lines, and whole insects

(A) Schematic of the experiment performed. Whole ovaries from nos>armRNAi or nos>arm^{S10} were dissected and DNA was extracted. Similarly, DNA was extracted from whole animals fed with Li^+ or insect cell lines treated with Li^+. Real time qPCR was performed on extracted DNA to quantify relative *Wolbachia* levels in the samples. (B) Reduction of Wnt signaling by knockdown of *armadillo* in the female germline led to a significant reduction in both *w*Mel and *w*MelCS levels. (C) Conversely, upregulation of Wnt signaling by expression of Arm^{S10} led to an increase in both *w*Mel and *w*MelCS levels. (D) Overexpression of Wnt signaling in insect cell lines by treatment with Li^+, a Wnt agonist, led to a significant increase in *Wolbachia* levels in *Drosophila melanogaster* (JW18), *Aedes aegypti* (Aag2) and *Aedes albopictus* (Aa23) cell lines. (E) Finally, feeding of Li^+ to *Drosophila melanogaster* was sufficient to increase *Wolbachia* (*w*Mel) levels in whole flies. *Wolbachia* levels were assessed by qPCR ratio of *Wolbachia* gene (wsp) over host DNA. *$p<0.05$; **$p<0.01$; ***$p<0.001$, errorbars show SEM.

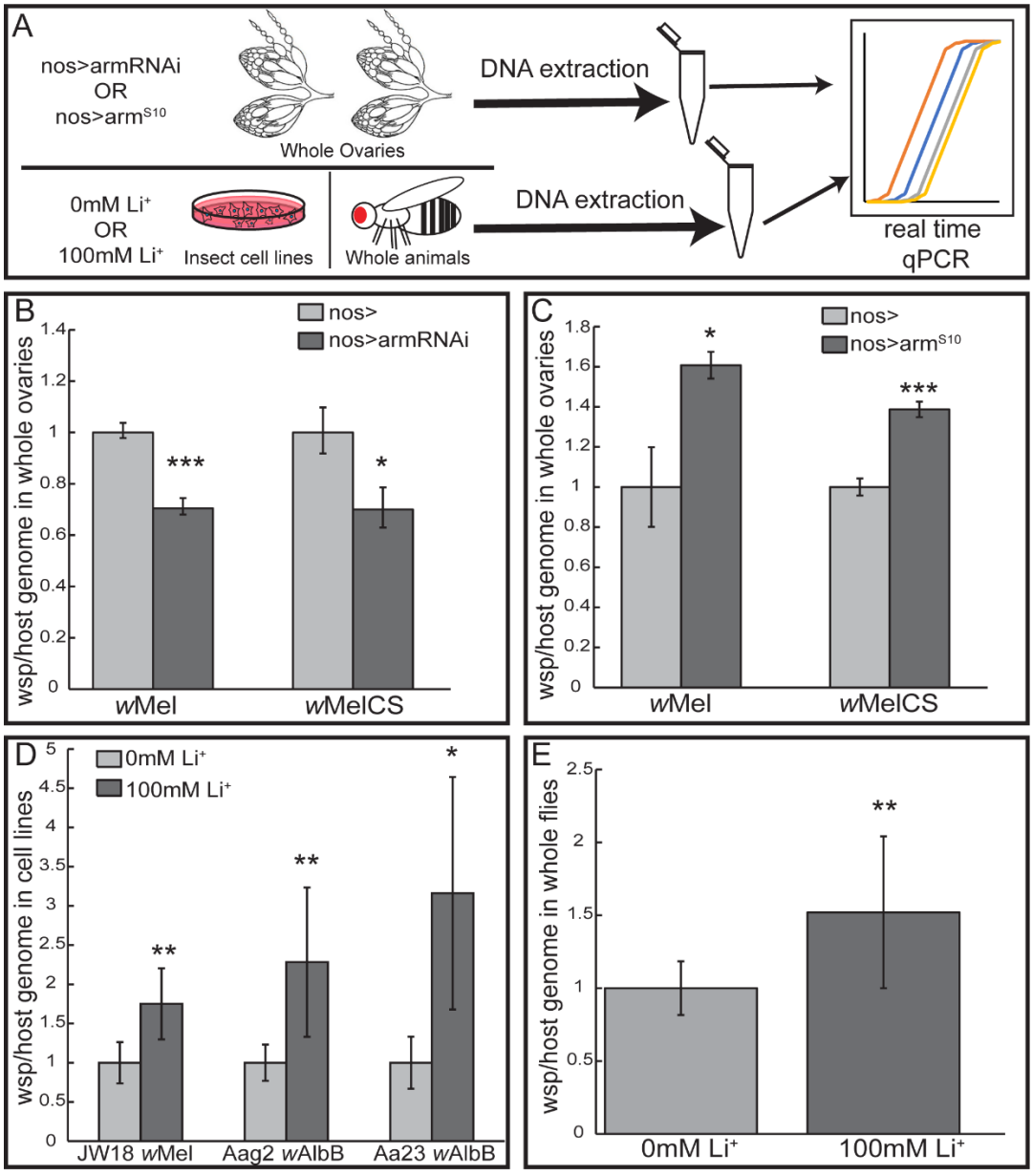

A
nos>armRNAi
OR
nos>armS10
Whole Ovaries
DNA extraction
0mM Li+
OR
100mM Li+
Insect cell lines
Whole animals
DNA extraction
real time
qPCR
B
nos>
nos>armRNAi
wsp/host genome in whole ovaries
wMel
wMelCS
C
nos>
nos>armS10
wsp/host genome in whole ovaries
wMel
wMelCS
D
0mM Li+
100mM Li+
wsp/host genome in cell lines
JW18 wMel
Aag2 wAlbB
Aa23 wAlbB
E
wsp/host genome in whole flies
0mM Li+
100mM Li+

Figure 4.10: Li^+ treatment leads to phosphorylation of Shaggy(sgg)

Western blotting of proteins extracted from flies fed with Li^+ showed an increased level of phosphorylated Shaggy (inactive form) compared to control flies which were fed normal food. Western blot against total Sgg showed no change in total protein upon Li^+ treatment.

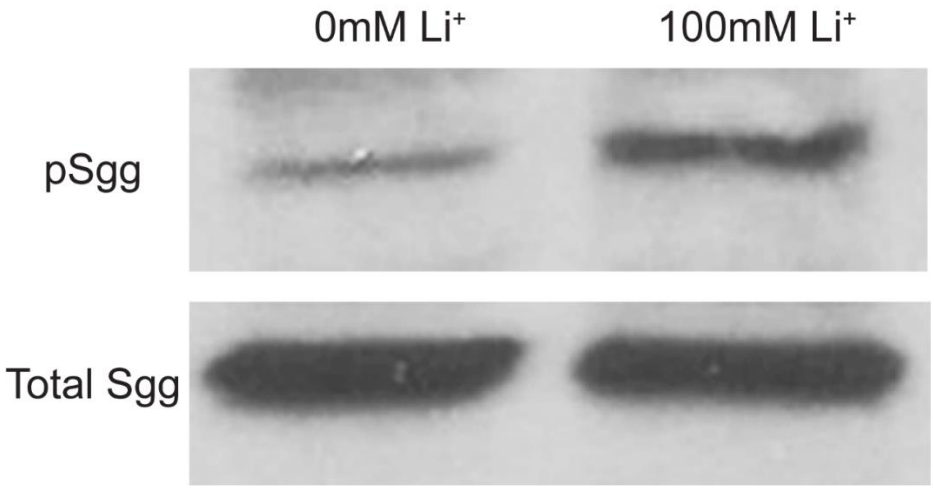
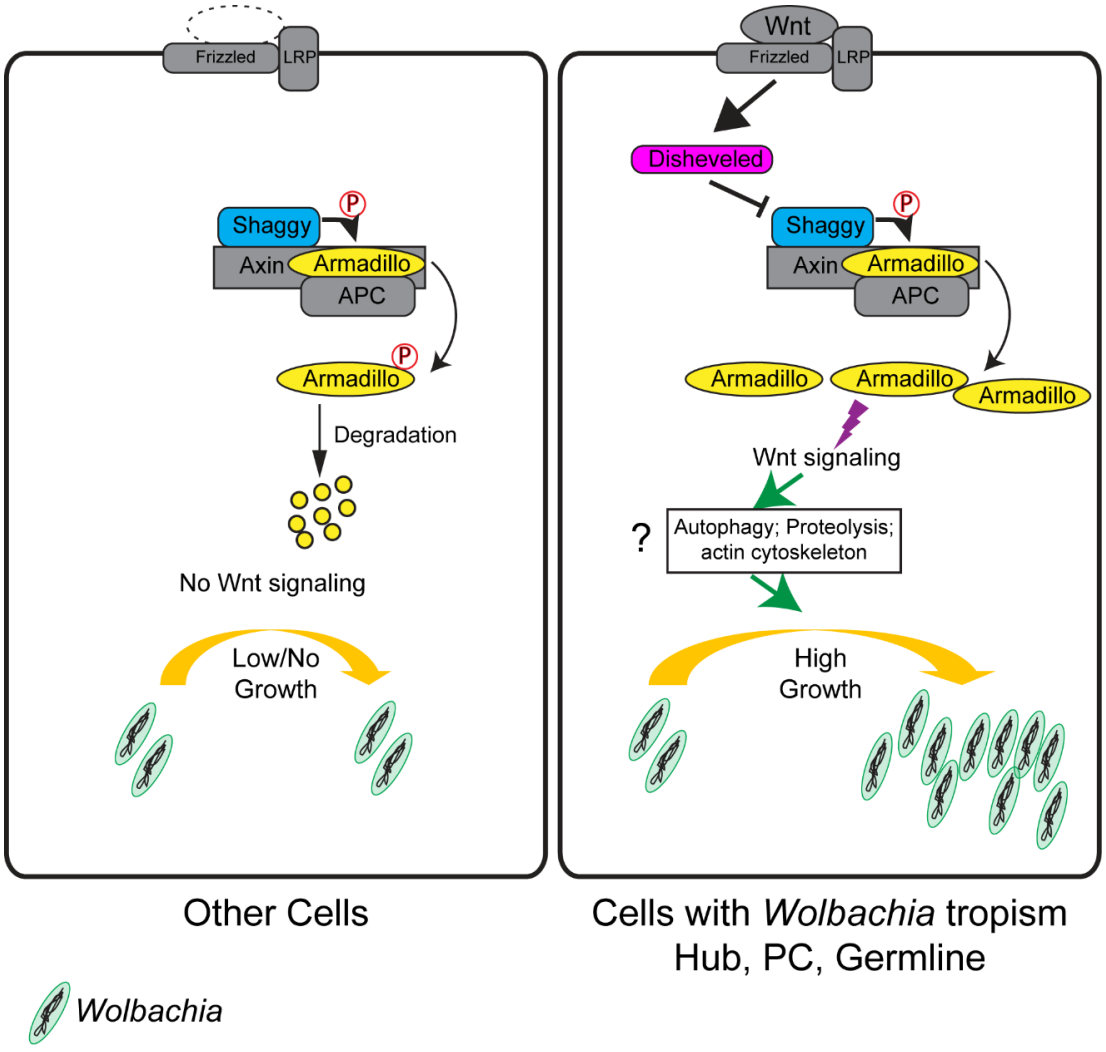


Figure 4.11: Model for how Wnt signaling induces *Wolbachia* tropism.

In cells with *Wolbachia* tropism including the hub, PCs and female germline, Wnt ligand activates Wnt signaling (right). This in turn modulates certain cell processes possibly including autophagy, proteolysis and actin cytoskeleton that will induce high *Wolbachia* intracellular growth. Conversely, in other cells (left), lack of Wnt signaling does not induce *Wolbachia* growth leading to lack of accumulation and tropism.



CHAPTER 5 DISCUSSION AND FUTURE DIRECTIONS

5.1 Summary of Findings

Being maternally transmitted, *Wolbachia* are under selective pressure to infect the female germline at high densities. However, there are multiple somatic tissues that are infected by *Wolbachia* (Pietri *et al.* 2016). These somatic tissues infected by *Wolbachia* have a consequence for both horizontal and vertical transmission of the bacteria. However, there is a lack of knowledge about the molecular and cellular mechanisms of *Wolbachia* tropism to specific host tissues. In this dissertation, we explore the molecular events that affect *Wolbachia* tissue tropism.

In Chapter 3, we identified a novel somatic cell tropism of *Wolbachia*. We surveyed 10 *Drosophila*-*Wolbachia* pairs and identified *Wolbachia* tropism to the polar cells (PCs), a specialized cell type in the *Drosophila* ovary. We further characterized the kinetics of *Wolbachia* accumulation to PCs and showed that *Wolbachia* coordinate their intracellular accumulation with specific events in PC morphogenesis. Being an obligate symbiont, *Wolbachia* need to coordinate their replication with host developmental events to maintain their density in rapidly growing host cells. At the same time, they need to maintain their levels to prevent host cell lysis. We found the pathogenic strain of *Wolbachia*, *w*MelPop, which kills infected flies by overreplicating and lysing cells (Min and Benzer 1997, Chrostek and Teixeira 2015), has lost this coordination in the PCs (Kamath *et al.* 2018). Moreover, using ectopic PCs, we show that PC specification is a sufficient trigger for *Wolbachia* to accumulate intracellularly. This shows that *Wolbachia* use certain developmental cues to coordinate their replication and intracellular accumulation.

Previously identified tropism to somatic cells such as the SSCN (Frydman *et al.* 2006) and GSCN (Toomey *et al.* 2013) were challenging to study during their development as these cells are formed during pupal stages and from a non-dedicated precursor population (Sahai-Hernandez and Nystul 2013). This novel *Wolbachia* PC tropism also paves way for identification of host signals that *Wolbachia* identifies to accumulate specifically.

In Chapter 4, we showed that Wnt signaling pathway controls *Wolbachia* tropism to *Drosophila* cell types and can modulate its intracellular density. Since Wnt signaling is essential in all somatic cell types infected with *Wolbachia* in *Drosophila* gonads, we surmised that this could be one of the cues utilized by the bacteria to grow intracellularly. Indeed, knocking down of Wnt signaling was sufficient to reduce *Wolbachia* density in infected cells and upregulation of Wnt signaling was sufficient to increase *Wolbachia* density. Moreover, we showed that upregulation of Wnt signaling in uninfected cell types could drive *Wolbachia* tropism to these cell types. We further showed that *Wolbachia* density could be increased by upregulating Wnt signaling using small molecule agonists of the pathway. We further replicated this effect in mosquito cell lines infected with other *Wolbachia* strains. These findings add to the growing literature that suggests a novel role for Wnt signaling in aiding intracellular growth of bacteria including *Mycobacterium tuberculosis* (Jayachandran *et al.* 2014, Villasenor *et al.* 2017), *Salmonella* (Liu *et al.* 2010), and *Ehrlichia chaffeensis*, a *Rickettsiales* bacteria like *Wolbachia* (Luo *et al.* 2015). Moreover, we demonstrate an ability to modulate *Wolbachia* density in non-model insects including mosquitoes using small molecule inhibitors. This will aid in efforts to introduce stable *Wolbachia* infections at high density in various insects that are vectors of devastating

diseases like Dengue, Zika virus, West Nile Virus, and Chikungunya. *Wolbachia* has been shown to be an effective way to control disease transmission by these vectors (Sinkins 2013, Aliota *et al.* 2016, Schultz *et al.* 2017). However, this effect by *Wolbachia* is density dependent (Osborne *et al.* 2009, Frentiu *et al.* 2010, Lu *et al.* 2012, Osborne *et al.* 2012), and these findings provide a tool to control *Wolbachia* density using small molecules will be extremely useful.

5.2 Significance

In this thesis, we have identified novel molecular mechanisms of *Wolbachia* tissue tropism to specific host cell types. The reliance of *Wolbachia* on host Wnt signaling provides a better understanding of *Wolbachia* tissue tropism in its hosts. Understanding the basic biology of how bacteria identify suitable host cells for infection has widespread implications. Several human pathogens have been found to utilize host Wnt signaling to aid their intracellular growth including *Salmonella*, *Mycobacterium tuberculosis*, and *Ehrlichia*. The *Drosophila* model has been instrumental in understanding several aspects of biology including embryogenesis, morphogenesis, and multiple conserved signaling pathways including Wnt signaling. Using the *Drosophila-Wolbachia* model we can gain better understanding of how bacteria utilize host Wnt signaling to promote their intracellular survival and growth. Moreover, the findings from this thesis can be applied in vector disease control, where *Wolbachia* has been found to be instrumental in blocking disease transmission by insect vectors. Understanding the molecular mechanisms of *Wolbachia* tissue tropism will be essential to understand this effect and provide us novel ways to augment these phenotypes.

5.2.1 Identification of coordination of *Wolbachia* tropism with host development

In chapter 3 we demonstrate the coordination of *Wolbachia* accumulation with host developmental events. Many insects carry obligate symbionts that are essential to shape their development (McFall-Ngai and Ruby 1991, Fraune and Bosch 2010, Lee and Brey 2013). However, for successful symbiosis, the symbiont must also coordinate its replication with the host and not stress the host cells. Studies from worms, *Brugia malayi* have shown that *Wolbachia* segregate unequally during embryogenesis and accumulate only in certain lineages (Landmann *et al.* 2010, Landmann *et al.* 2012). These show that *Wolbachia* have evolved mechanisms to coordinate their accumulation with specific host events. The coordination of *Wolbachia* accumulation with PC specification and development provides us a well characterized system that is easy to follow during morphogenesis which we can use to further dissect this aspect of *Wolbachia* tropism. Different *Wolbachia* strains distribute differently in embryos of their respective *Drosophila* hosts. For instance, *w*Ri distributes evenly in the embryo of *D. simulans*, whereas *w*Mel, *w*MelCS and *w*MelPop localize to the pole plasm of *D. melanogaster*, which forms the germline. Finally, *w*No and *w*Ma localize to the anterior of the egg, away from the pole plasm in *D. simulans* and *D. mauritiana* respectively (Veneti *et al.* 2004). All adults derived from these embryos however, have *Wolbachia* infection in the mature germline. PC studies will provide insights into the timing of *Wolbachia* accumulation with host developmental events and allow for further studies of how *Wolbachia* infect the germline during morphogenesis, eventually providing us insights into this neglected aspect of the host-symbiont interactions.

5.2.2 Host Wnt signaling drives *Wolbachia* tropism

Analyzing various somatic cells infected by *Wolbachia* in *Drosophila* gonads allowed us to characterize the role of Wnt signaling in *Wolbachia* tropism. Wnt signaling was first characterized as a conserved pathway that controls embryogenesis, morphogenesis, stem cell division, cell-fate specification, and cell proliferation (DasGupta *et al.* 2005, Bejsovec 2013). However, novel roles of Wnt signaling in immunity and hematopoiesis have been discovered in the past few years (Staal and Clevers 2005, Staal *et al.* 2008). The reliance of *Wolbachia* on Wnt signaling provides us with many insights into the biology of intracellular bacteria. Moreover, these findings provide us with applications in vector disease control. Both these are discussed in detail below.

5.2.2.1 Identification of the role of Wnt pathway in *Wolbachia* tropism

Wnt signaling is implicated in phagocytosis and intracellular survival of viruses and bacteria (Maiti *et al.* 2012, Zhu and Zhang 2013). Studies have shown that intracellular pathogenic bacteria interact with host Wnt signaling to promote their survival. *Salmonella*, a gastrointestinal pathogen, secretes effectors to upregulate β-catenin/Armadillo (Arm) in intestinal stem cells thereby upregulating Wnt signaling. This leads to high stem cell activity in infected intestines (Sun *et al.* 2004, Ye *et al.* 2007, Liu *et al.* 2010). *Wolbachia* infection has been shown to increase stem cell activity in infected flies leading to increased rate of egg production (Fast *et al.* 2011). These effects by *Salmonella* and *Wolbachia* could be mediated by similar mechanisms. *Mycobacterium tuberculosis*, infect macrophages and upregulate non canonical Wnt signaling leading to increase in Ca^{+2} signaling, eventually blocking the fusion of phagosomes (containing *Mycobacteria*) with lysosomes

(Blumenthal *et al.* 2006, Jayachandran *et al.* 2014, Villasenor *et al.* 2017). Similarly, *Ehrlichia*, close cousins of *Wolbachia*, infect macrophages and increase Wnt signaling to promote phagocytosis of more bacteria and block the fusion of lysosome and phagosomes (Luo *et al.* 2015, Mitra *et al.* 2018). These reports, along with reports suggesting the regulation of *Wolbachia* by autophagy (Voronin *et al.* 2012), suggest that *Wolbachia* would highly infect cells with high Wnt signaling, as shown by our data. This role of Wnt signaling in *Wolbachia* tropism adds to the growing literature of bacterial interaction with host Wnt signaling. Moreover, *Drosophila* gonads provide us with a model system to study the reliance of intracellular bacteria on Wnt signaling. These studies would be invaluable in furthering the understanding of this novel role of Wnt signaling in intracellular bacterial survival.

5.2.2.2 Applications in vector disease control

The utilization of *Wolbachia* is increasingly emerging as a novel tool to control the vector competency of insects that spread devastating human diseases like Dengue, Zika virus, and Chikungunya (Blagrove *et al.* 2013, Sinkins 2013, Caragata *et al.* 2016, Schultz *et al.* 2017). *Wolbachia* infection of vectors leads to viral inhibition thereby leading to inhibition of disease transmission to humans.

However, there are certain limitations to the widespread application of this to all vectors. In the mosquito *Aedes albopictus*, natural strains of *Wolbachia* have low bacterial loads in somatic tissues thereby preventing efficient viral inhibition (Lu *et al.* 2012). Moreover, studies have used antibiotics to reduce *Wolbachia* density in hosts including *Drosophila simulans* (Osborne *et al.* 2009, Osborne *et al.* 2012) and mosquito cell lines

(Schultz *et al.* 2017). In both cases, reduction of *Wolbachia* density leads to increase in viral titers. These suggest that *Wolbachia* density is important in blockage of viral transmission.

Another challenge is the inability of certain vectors to harbor *Wolbachia*. *Wolbachia* infection of *Anopheles gambiae* inhibits Plasmodium, however the *Wolbachia* infection is transient and is lost eventually (Hughes *et al.* 2011). The native microbiome is implicated as a factor inhibiting *Wolbachia* maternal transmission in these mosquitoes (Hughes *et al.* 2014). Moreover, certain strains of *Wolbachia* such as *w*Str show stronger potential to inhibit viral growth in mosquito cell lines (Schultz *et al.* 2017).

Our results show that small molecule Wnt agonists are sufficient to increase *Wolbachia* levels in cell lines and whole animals. Upregulating Wnt signaling in cell lines while infecting them with novel *Wolbachia* strains may be an approach to obtain high intracellular densities and establish these *Wolbachia* strains in these hosts. Further, activating Wnt signaling using transgenic mosquitoes to drive high levels of *Wolbachia* tropism to the female germline could ensure maternal transmission and establishment of *Wolbachia* in the population. It would, however, be important to address any adverse effects of high Wnt signaling in these mosquitoes before considering them for disease control.

The Anti-*Wolbachia* consortium (A-WOL) is working to develop tools to target *Wolbachia* in parasitic filarial nematodes as a treatment for filariasis (Slatko *et al.* 2010)(www.a-wol.com). These filarial worms depend on *Wolbachia* for reproduction, growth, and survival. Elimination of *Wolbachia* from these makes them sterile and shortens

their lifespan making it a convenient target in filarial infections (Taylor and Hoerauf 1999, Taylor 2000, Taylor *et al.* 2000, Slatko *et al.* 2010). These observations suggest that Wnt signaling, being conserved from worms to insects, is involved in *Wolbachia* growth in filarial worms as well. This would provide us with another class of drugs, Wnt inhibitors, that could be used in conjunction with doxycycline in the A-WOL effort. Moreover, given the extensive characterization of Wnt signaling in various cancers, there exist multiple small molecule agonists and antagonists that can be rapidly applied to these efforts (Meijer *et al.* 2004, Barker and Clevers 2006, Nusse and Clevers 2017).

5.3 Future directions – possible mechanisms of Wnt pathway mediated *Wolbachia* intracellular growth

Being maternally transmitted, *Wolbachia* have high tropism to the female germline. However, *Wolbachia* have tropism to specific somatic cells of the *Drosophila* gonads including the male and female stem cell niches (Frydman *et al.* 2006, Toomey *et al.* 2013, Toomey and Frydman 2014) and the female polar cells (PC) (Kamath *et al.* 2018). These somatic cell types have proven to be useful in elucidating the molecular mechanisms of *Wolbachia* tropism. Our research suggests that the host Wnt signaling pathway controls *Wolbachia* tropism to various cell types and affects *Wolbachia* density.

Wnt pathway is a well conserved pathway involved in embryonic development and cell proliferation (DasGupta *et al.* 2005, Bejsovec 2013). The role of Wnt pathway has now been described in many other cellular processes including stem cell division (Willert *et al.* 2003), immunity (Staal *et al.* 2008, Zhu and Zhang 2013, Silva-Garcia *et al.* 2014), autophagy (Jansson *et al.* 2005, Jiang *et al.* 2009, Petherick *et al.* 2013, Li *et al.* 2016), and

proteasome mediated degradation (DasGupta *et al.* 2005, Petherick *et al.* 2013, Stamos and Weis 2013) among others. The activation of Wnt signaling triggers diverse downstream responses in cell specific manner. However, the cell processes affected by Wnt signaling in the hub, PCs, and female germline have not been well characterized. We want to further characterize which host molecules affect *Wolbachia* intracellular densities in these cell types and how they are modulated by Wnt signaling.

The next question is which host molecules interact with *Wolbachia* to induce their intracellular growth in these cells? Future studies will aim to identify which processes downstream of Wnt signaling would affect *Wolbachia* density in *Drosophila* gonads. These studies can be broken down into multiple questions: 1) Which specific downstream genes are modulated by canonical Wnt signaling in these *Wolbachia* infected cell types; 2) Does host proteolysis affect *Wolbachia* levels upon activation of Wnt signaling; 3) Does Wnt signaling affect rates of autophagy in these cell types? Below, we discuss these questions in more detail.

5.3.1 Which genes are affected by Wnt signaling in *Wolbachia* infected cells?

5.3.1.1 Pangolin is a Wnt specific transcription factor that co-activates signaling with Armadillo.

Upon activation of Wnt signaling, cytoplasmic Arm levels increase and it translocates to the nucleus where it binds with Pangolin (Pan), a Wnt specific transcription factor, leading to transcription of Wnt responsive genes (van de Wetering *et al.* 1997, Schweizer *et al.* 2003, Bejsovec 2013, Archbold *et al.* 2014). To determine whether transcription of Wnt responsive genes through Pan is necessary for *Wolbachia*

accumulation, we can express a dominant negative mutant of Pan, PanΔN. This mutant lacks the Arm binding domain leading to an inactivation of Wnt responsive genes in a dominant negative manner (van de Wetering *et al.* 1997, Archbold *et al.* 2014). Expressing PanΔN in hubs and PCs independently and epistatically with Arm^{S10} would serve to determine whether transcription of downstream genes of Wnt canonical signaling is required for *Wolbachia* accumulation or whether some transcription independent function of Arm is at play here. Our preliminary results shown in Appendix D suggest that Pangolin mediated transcription is not necessary for *Wolbachia* growth in hubs and PCs. Further analyses will need to be performed to confirm these results and investigate other Wnt specific co-transcription factors necessary for *Wolbachia* growth

5.3.1.2 Whole genome transcriptome analysis

Expression of Arm^{S10} in the female germline under *nos*-Gal4 control is sufficient to induce Wnt signaling and increase *Wolbachia* levels. Whole genome transcriptome analysis by RNAseq would help identify Wnt downstream genes that would be dysregulated in this system allowing us to further characterize host processes that would explain *Wolbachia* modulation by host Wnt signaling.

5.3.2 Interaction of proteasome mediated proteolysis with active Wnt signaling

In vertically transmitted endosymbionts, there is a strong selective pressure for genome reduction. Reports from genome analyses show that *Wolbachia*, along with other *Rickettsiales* bacteria lack biosynthetic pathways for amino acids (Wu *et al.* 2004, Dunning Hotopp *et al.* 2006). However, *Wolbachia* retain amino acid uptake transporters, and amino acid metabolism pathways suggesting that they use host amino acids as an energy source

(Dunning Hotopp *et al.* 2006). Some studies show that host proteasome is activated by *Wolbachia* infection causing degradation of host proteins leading to increased availability of amino acids to support establishment and maintenance of *Wolbachia* (Fallon and Witthuhn 2009, Zheng *et al.* 2011). Consistent with these, knockdown of proteasome activity in *Wolbachia* infected cells leads to reduction of infection (White *et al.* 2017). Considering these findings, we can hypothesize that upon activation of Wnt signaling, rate of host proteolysis would be elevated leading to a favorable environment for *Wolbachia* growth.

5.3.2.1 Determining proteolysis rates under active Wnt signaling

To determine whether high Wnt signaling promotes high proteasome activity, we can conduct a proteasome activity assay on ovaries expressing Arm^{S10}, a constitutively active Arm, in their germline under the control of *nos*-Gal4. Using a fluorogenic substrate, Z-LLE-AMC, proteasome activity can be measured in protein lysates from whole ovaries.

Preliminary results highlighted in Appendix E suggest that proteasome activity is increased in ovaries with active Wnt signaling. This suggests that activation of canonical Wnt signaling would increase the pool of free amino acids, thus creating a favorable environment for *Wolbachia* to grow intracellularly. We can further characterize proteolysis by studying the Ubiquitination profile of proteins extracted from these ovaries. Activation of proteolysis would increase the general ubiquitination of proteins and can be quantified by western blot analysis.

5.3.2.2 Modulation of host proteolysis to study effect on *Wolbachia*.

High rates of host proteolysis have been shown to be required for *Wolbachia* intracellular growth in *Drosophila* and mosquito cell lines (Fallon and Witthuhn 2009, White *et al.* 2017). We could determine the effect of proteolysis in *Drosophila* gonads by genetic and chemical means. Overexpression of Ubiquitin would be sufficient to increase proteolysis of intracellular proteins (Daino *et al.* 2000, Kutty *et al.* 2005, Crinelli *et al.* 2008, Lee *et al.* 2009). Overexpression of Ubiquitin can be done using the Gal4-UAS system in the female germline under *nos*-Gal4 control. Conversely, knockdown of proteolysis can be done by knocking down the E1 ligase Uba1 (Chang *et al.* 2013) or E2 ligase Ubc6 (Chen *et al.* 2011) using RNAi. Moreover, use of proteasome inhibitor drugs like MG132 and epoxomicin can be used to reduce host proteolysis (Velentzas *et al.* 2011). Determining *Wolbachia* density in these experiments independently or epistatically with Arm^{S10} would allow us to dissect the role of proteolysis in Wnt signaling mediated *Wolbachia* tropism.

5.3.3 Modulation of autophagy by Wnt signaling to promote *Wolbachia* growth

Ehrlichia, a human pathogen, upregulates the Wnt pathway in macrophages to block host autophagy, specifically phagosome-lysosome fusion, to promote their own survival (Luo *et al.* 2015, Mitra *et al.* 2018). Moreover, *Wolbachia* intracellular survival is dependent on host autophagic response (Voronin *et al.* 2012, Serbus *et al.* 2015). Wnt signaling promotes cell proliferation and differentiation (Bejsovec 2013) and is shown to inhibit both basal and stress related autophagy in multiple systems (Jansson *et al.* 2005, Jiang *et al.* 2009, Petherick *et al.* 2013, Fu *et al.* 2014, Li *et al.* 2016). More specifically,

Wnt signaling blocks the formation of autolysosomes (Petherick *et al.* 2013). Considering these studies, we can hypothesize that high Wnt signaling would inhibit autophagy leading to increased *Wolbachia* growth. Future experiments will aim to elucidate this by studying the different forms and localizations of various autophagy specific proteins such as ATG8a and Ref(2)p in presence of Arm^{S10} by western blots and IHC analyses. Moreover, using RNAi and drugs to inhibit autophagy epistatically with active Wnt signaling would further dissect the role of autophagy in Wnt signaling mediated *Wolbachia* tropism.

5.4 Conclusion

The cellular and molecular mechanisms of *Wolbachia* tissue tropism are not well characterized. This thesis has explored novel *Wolbachia* tropism to somatic cells in *Drosophila* gonads as well as shed light on the coordination of *Wolbachia* intracellular accumulation with host developmental events. Moreover, we have shown the dependence of *Wolbachia* on a conserved host signaling pathway, Wnt signaling. This adds to the growing literature exploring the role of Wnt signaling in intracellular bacterial growth (Tolwinski and Wieschaus 2004, Staal *et al.* 2008, Maiti *et al.* 2012) and provides a targetable pathway to modulate *Wolbachia* levels in non-model arthropods. Reduction of vector competence by *Wolbachia* is an emerging tool to control vector transmitted diseases (Moreira *et al.* 2009, Kambris *et al.* 2010, Bian *et al.* 2013, Caragata *et al.* 2016, Schultz *et al.* 2017). Understanding *Wolbachia* tropism and interaction with the host will be invaluable in developing new *Wolbachia*-based approaches as well as augmenting current approaches.

Appendix A: Identification of various *Wolbachia* strains using Multilocus Sequence Typing (MLST)

Different *Wolbachia* strains that infect various insects can be differentiated based on their genomes. The most widely used method to differentiate *Wolbachia* strains is by Multilocus Sequence Typing (MLST) which was developed by Paraskevopoulos *et al*. (Paraskevopoulos *et al.* 2006) and Baldo *et al.* (Baldo *et al.* 2006). MLST is based on direct nucleotide sequencing of a target locus, to which a unique and arbitrary allele number is assigned. Upon sampling multiple target genes, the combination of allele numbers for each isolate is defined as the allelic profile. The most appropriate MLST loci are housekeeping loci, which: (1) are ubiquitous within the population; (2) usually encode proteins that are essential for central metabolism; (3) typically evolve at a moderate rate; and (4) are subject to purifying selection.

Using loci corresponding to *dnaA*, *pdhb*, *wsp* and *16srdna* genetic regions of the genome, primers were designed as shown in Table A1.1. PCR was performed on various *Drosophila melanogaster* stocks and resulting product of each gene was sequenced. Based on the MLST profiles published by Baldo et al., the *Wolbachia* strains were typed as shown in Table A1.2. Three *Wolbachia* strains infecting *D. melanogaster*, *w*Mel, *w*MelCS and *w*MelPop all share the same MLST profiles. To differentiate them further, the Variable Number Tandem Repeats (VNTR) locus for the "*w*Mel" infected flies was sequenced. Based on Riegler et al., *w*Mel and *w*MelCS have a different size of VNTR105 and VNTR141 loci (Riegler *et al.* 2012). Based on this, VNTR primers were designed (Table A1.3) and PCR was performed to get the size of the VNTR loci. The *w*Mel subgroup was

further differentiated into *w*Mel or *w*MelCS/*w*MelPop. The subgrouping is shown in Table A1.4. Further differentiation between *w*MelCS and *w*MelPop could not be done with this method.

Table A.1 Degenerate Primers used for MLST

Gene/Locus	Forward Primer	Reverse Primer	Amplicon size
dnaA	GGTCTCCTAGTGATCTTGATGGAG	TAACAGTAGCATGGTCTCTGCC	400bp
pdhb	ARGAAGTTGCVGARTATSAWGG	CACGTACAACTGGAGCATCAAG	750bp
16srdna	GTTGGAAACGGCAACTAATACC	CGAATTAAACCACATGCTCCAC	700bp
wsp	GTCCAATARSTGATGARGAAAC	CYGCACCAAYAGYRCTRTAAA	650bp

Primer degeneracy code:

R:A/G; Y:C/T; M:A/C; K:G/T; S:C/G; W:A/T; V:A/C/G.

Table A.2 *Wolbachia* strains determined by MLST

Frydman lab stock number	*Wolbachia* strain
7	*w*Mel
163	*w*Mel
216	*w*Mel
217	*w*Mel
201	*w*Mel
202	*w*Mel
177	*w*No; *w*Ma
191	*w*No; *w*Ma

Table A.3 Primers used for VNTR

Locus	Forward Primer	Reverse Primer
VNTR141	GGAGTATTATTGATATGCG	GACTAAAGGTTAGTTGCAT
VNTR105	GCAATTGAAAATGTGGTGC	ATGACACCTTACTTAACCGTC

Table A.4 *Wolbachia* strains determined by MLST and VNTR

Frydman lab stock number	*Wolbachia* strain
7	*w*MelCS/*w*MelPop
163	*w*Mel
216	*w*MelCS/*w*MelPop
217	*w*Mel
201	*w*Mel
202	*w*MelCS/*w*MelPop

Appendix B: Purification of polar cells from *Drosophila* ovaries for RNA extraction and sequencing.

In chapter 3, *Wolbachia* tropism to a novel cell type in the *Drosophila* ovary, the PC was characterized. To understand which host genes might affect *Wolbachia* in these cell types, RNAseq of these cells specifically need to be performed. To extract PC specific RNA, a new method of isolating PCs from whole ovaries was adapted from Wang *et al* (Wang *et al.* 2008).

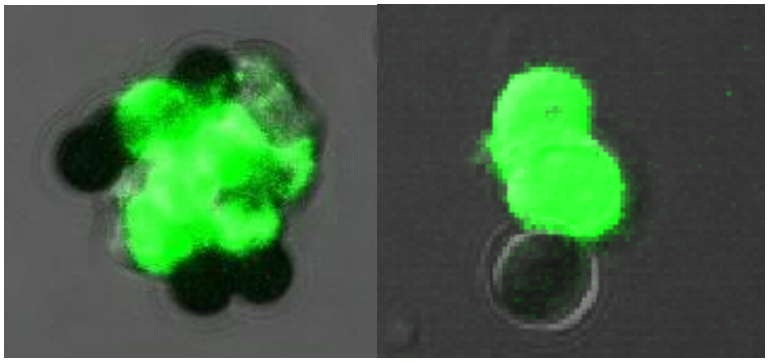
Using the Gal4/UAS system, mCD8-GFP was expressed on the membranes of PC. Seven-day old flies were dissected to obtain their ovaries. These ovaries were dissociated using 4mg/ml Elastase in a cell dissociation buffer (Sigma) for 40 mins. The dissociated cells were passed through a 40µm filter to filter out large germline cells. The remaining cells were incubated with magnetic bead conjugated antibodies against mCD8 for about 30 mins. The solution was kept on a magnet leading to the sequestration of the magnetic beads (bound to PC) to the walls of the tube. The remaining supernatant was removed and 2 washes were performed on the beads in the same way. After the final wash, the beads were dissociated from the PC using a high temperature of 56ºC and RNA extraction was performed on the purified PC. Some of the beads were stained for a DNA dye (Hoechst) and mounted on a slide. Number of GFP+ and GFP- cells were counted and percent purity of the samples was assessed as shown in Table A2.1

Table B.1 Polar Cell extraction purity using mCD8-GFP

Cells	GFP status	Number
Polar Cells	GFP+	103
Lateral follicle cells	GFP-	47
Total	GFP+, GFP-	150
Percentage purity of Polar Cells (GFP+/Total)		**69%**

Figure B.1 Polar cells bound to magnetic beads

Shown here are images of polar cells expressing mCD8-GFP (green) bound to magnetic beads conjugated to anti-mCD8 antibodies (black circles)



Appendix C: Effect of *Wolbachia* on JAK-STAT pathway in the hub

C.1 JAK-STAT pathway is required for testis stem cell division

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway was discovered to modulate stem cell activity in the *Drosophila* testis. The cytokine Unpaired (Upd) is secreted by hub cells that binds to its receptor, Domeless, in GSCs and CySCs (Harrison *et al.* 1998, Matunis *et al.* 2012). STAT (Signal transducer and activator of transcription) is a downstream effector that translocates into the nucleus upon activation of signaling and activates transcription of STAT responsive genes (Fig.C.1) (Hombria and Brown 2002). JAK-STAT signaling is required in the stem cells for their maintenance, as depletion of STAT results in loss of stem cells (Matunis *et al.* 2012). GSC and CySC replication needs to be coordinated in a ratio of 2:1 to have accurate sperm production (Matunis *et al.* 2012). For this, Upd synthesis in hub cells is finely regulated by siRNAs and miRNAs. *upd* mRNA is degraded by siRNAs and is stabilized by a IGF-II mRNA binding protein (IMP). *imp* mRNA levels are further maintained by degradation by a miRNA let-7 (Toledano *et al.* 2012).

Previously, *Wolbachia* has been shown to regulate miRNA levels upon infection (Hussain *et al.* 2011, Zhang *et al.* 2013). One of the miRNAs affected by *Wolbachia* is the *let-7* miRNA (Hussain *et al.* 2011). As *Wolbachia* infect hub cells at high density (Toomey and Frydman 2014), we wanted to investigate the effect of *Wolbachia* on the miRNA and JAK-STAT activity in the testis' hubs.

C.2 *Wolbachia* infection leads to reduced levels of *upd* and *imp* in whole *Drosophila* testis

In the *Drosophila* testis, there is *upd* expression only in the hubs. To determine whether *upd* mRNA levels were changed, we extracted total RNA from whole testis and performed qRT-PCR. Upon analysis we observed that *upd* mRNA levels in *w*Mel infected testis were two-fold lower than *w*- testis. Similarly, *w*MelCS infected testis had about 2.5-fold lower *upd* levels than *w*- testis (Fig. C.2A). To explore the mechanism of *upd* mRNA downregulation, we probed for *imp* transcript levels in whole testis. We observed a reduction in *imp* transcript levels of about 1.4-fold in *w*Mel and about two-fold in *w*MelCS infected testis (Fig. C.2B).

As IMP stabilizes *upd* mRNA, reduction in IMP levels will cause a reduction in *upd* mRNA levels. The investigate the mechanism further, we need to determine whether levels of *let-7* miRNA are changed in *Wolbachia* infected testis.

C.3 *Wolbachia* infection leads to reduction in JAK-STAT signaling

Upd activates downstream JAK-STAT signaling in cells with the ligand Domeless. Studies indicate that Upd secreted by the hubs activates JAK-STAT signaling in GSCs and CySCs. To study the activation of JAK-STAT signaling, we introduced a 10X-STAT-GFP into our flies. This construct expresses GFP in cells with active JAK-STAT signaling. We expected high levels of GFP in the GSCs and CySCs, however we observed a high GFP expression in the hub cells with a diffuse staining in GSCs and CySCs. We quantified the GFP levels in the hub in *w*Mel and *w*MelCS infected hubs and compared it to GFP levels

in uninfected hubs. We did observe a significant decrease in GFP level in both *Wolbachia* infected hubs compared to *Wolbachia* uninfected hubs (Fig.C.3).

These results mean that there is some autocrine JAK-STAT signaling in the testis' hub and it is dysregulated by *Wolbachia*. However, we cannot rule out the possibility of false positives generated by the reporter. These data suggest a role for *Wolbachia* affecting host phenotypes by microRNAs that need to be characterized in further detail. Future studies should be performed with other reporters as well as to test the levels of microRNAs directly.

Figure C.1 JAK-STAT pathway in *Drosophila* testis

The hub secretes the ligand Unpaired (Upd), which activates JAK-STAT signaling by binding to the receptor, Domeless (purple). The signaling propagates through Hopscotch, JAK and STAT resulting in activation of downstream genes in the GSC (studied here using a 10XSTAT-GFP construct). In the hub, Upd levels are carefully controlled by siRNAs. The transcription of the *upd* mRNA is inhibited by siRNAs. IMP (IGF II mRNA binding protein) stabilizes the *upd* mRNA and prevents inhibition by siRNAs. The transcription of the *imp* mRNA is in turn regulated by the conserved miRNA *let-7*.

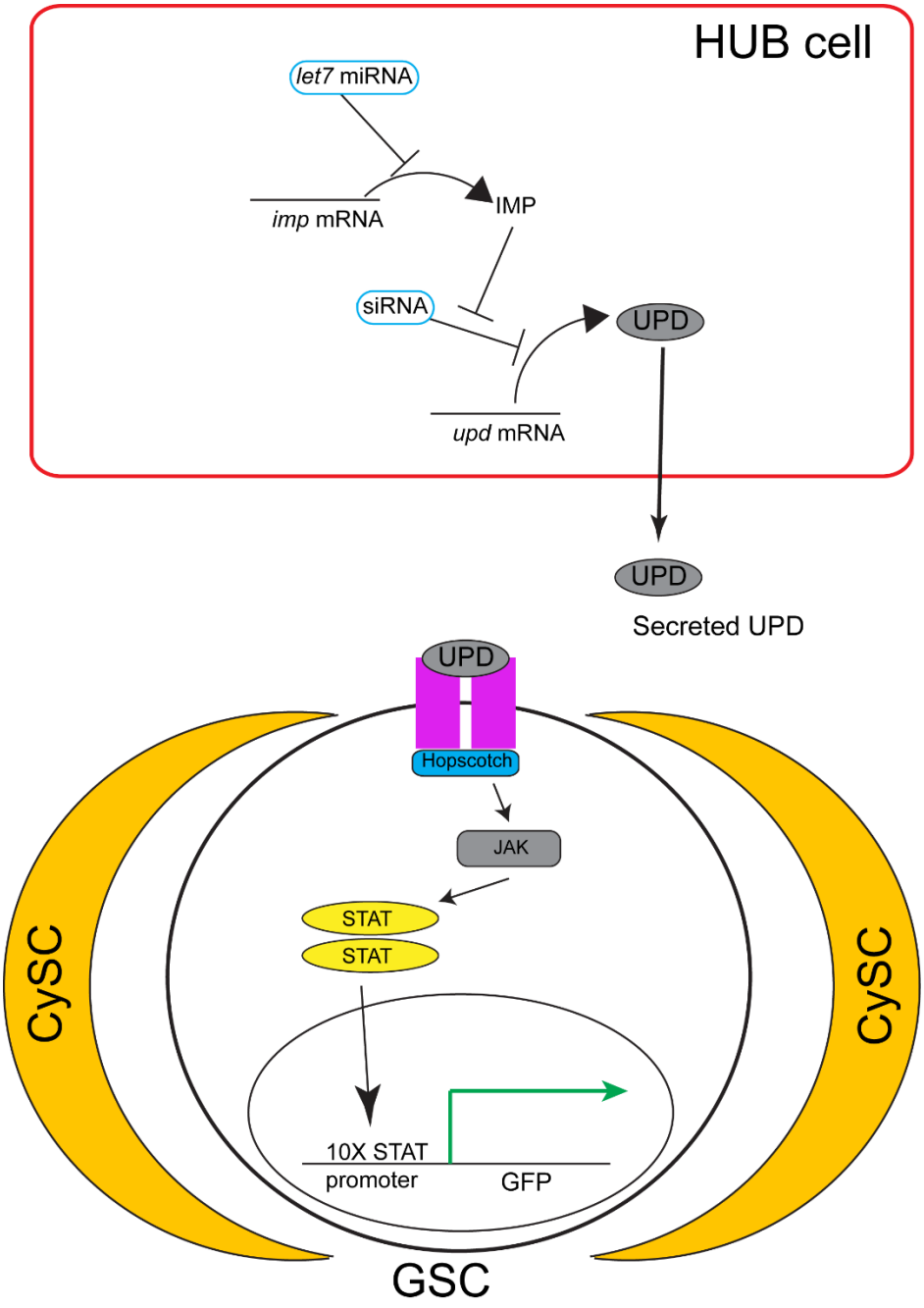

HUB cell
let7 miRNA
imp mRNA
IMP
siRNA
UPD
upd mRNA
UPD
Secreted UPD
UPD
Hopscotch
JAK
STAT
STAT
CySC
CySC
10X STAT
promoter
GFP
GSC

Figure C.2 *Wolbachia* causes reduction in *upd* and *imp* mRNA in whole testis

(A) shows mRNA levels of *upd* in whole *Drosophila* testis quantified by qRT-PCR. *w*Mel and *w*MelCS infected testis have significantly reduced upd levels compared to w- testis. (B) shows *imp* mRNA levels in whole *Drosophila* testis. There is a reduction in *imp* levels in both *w*Mel and *w*MelCS infected testis. however, only the *w*MelCS levels are statistically significantly reduced. *p<0.05, Student's t test.

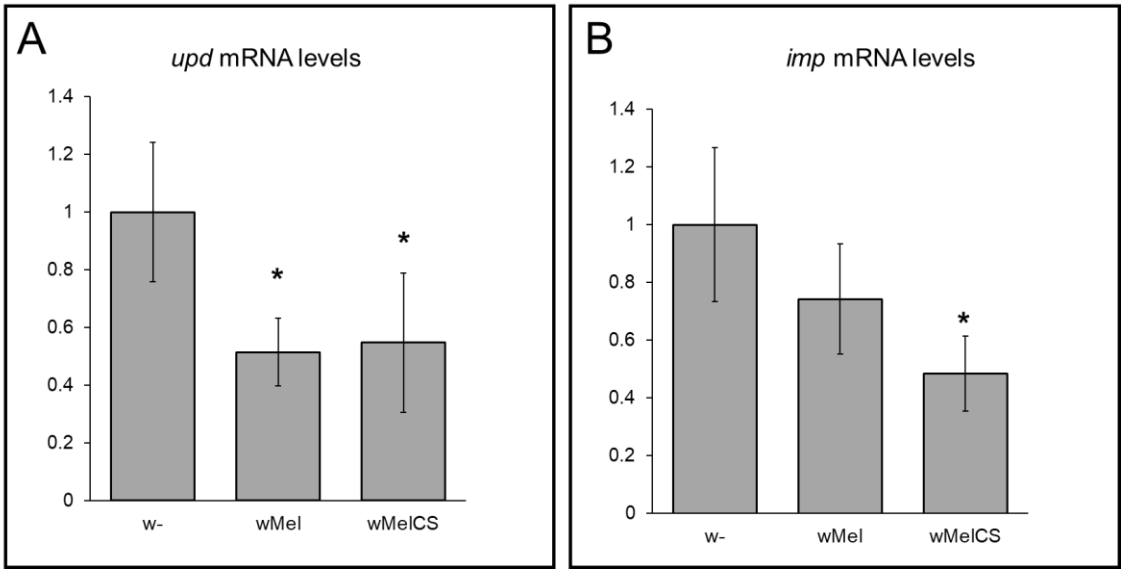
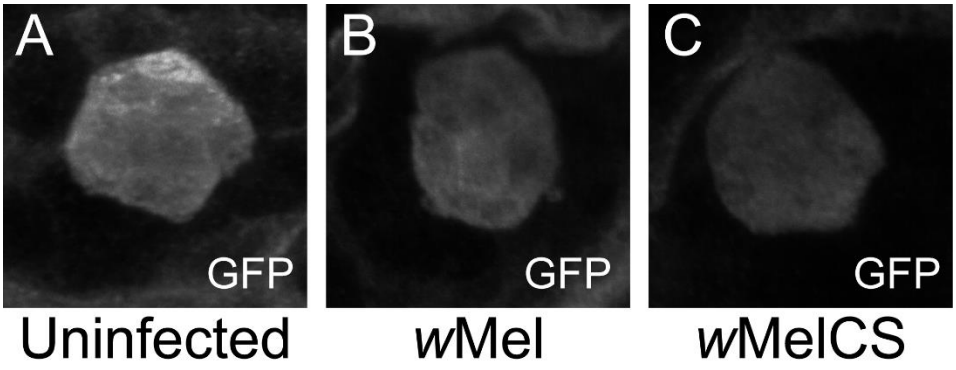
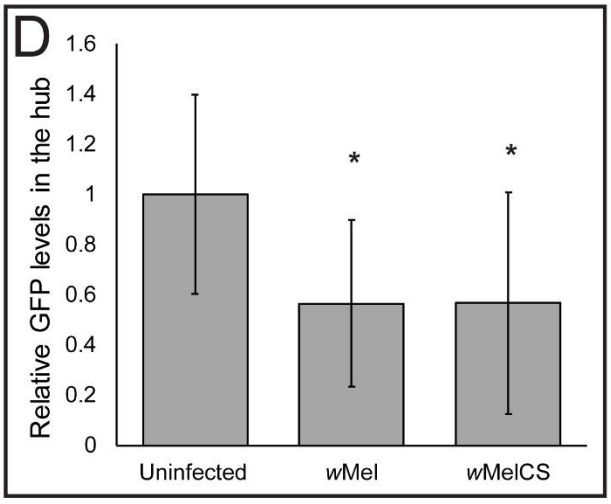


Figure C.3 *Wolbachia* infection leads to reduction of JAK-STAT activity in *Drosophila* hubs

Confocal images of hubs with DNA stained in blue, a hub marker (Armadillo) in Red and GFP (10XSTAT-GFP) marked in Green. Uninfected hubs (A,A') have slightly higher GFP intensity compared to wMel (B,B') or wMelCS (C,C') infected hubs. GFP levels were quantified in the hubs and normalized to uninfected GFP levels. (D) Quantification shows that *Wolbachia* infection leads to a two-fold reduction in GFP (JAK-STAT activity) in the hubs. *p<0.05, Student's t test.





Appendix D: Transcription of Armadillo through Pangolin (Pan) does not affect *Wolbachia* density

In cells with active Wnt signaling, Arm translocates to the nucleus and binds to Pan, a Wnt specific transcription factor, to activate gene transcription of Wnt responsive genes (van de Wetering *et al.* 1997, Schweizer *et al.* 2003, Bejsovec 2013, Archbold *et al.* 2014). To determine whether transcription of Wnt responsive genes through Pan is necessary for *Wolbachia* accumulation, we expressed a dominant negative mutant of Pan, PanΔN. Due to the lack of Arm binding domain, PanΔN causes inactivation of Wnt responsive genes in a dominant negative manner (van de Wetering *et al.* 1997, Archbold *et al.* 2014). We hypothesize that Arm^{S10} upregulates *Wolbachia* density through Pan dependent transcription of Wnt signaling. Upon expression of PanΔN in cells expressing Arm^{S10}, we would expect a reduction in *Wolbachia* density.

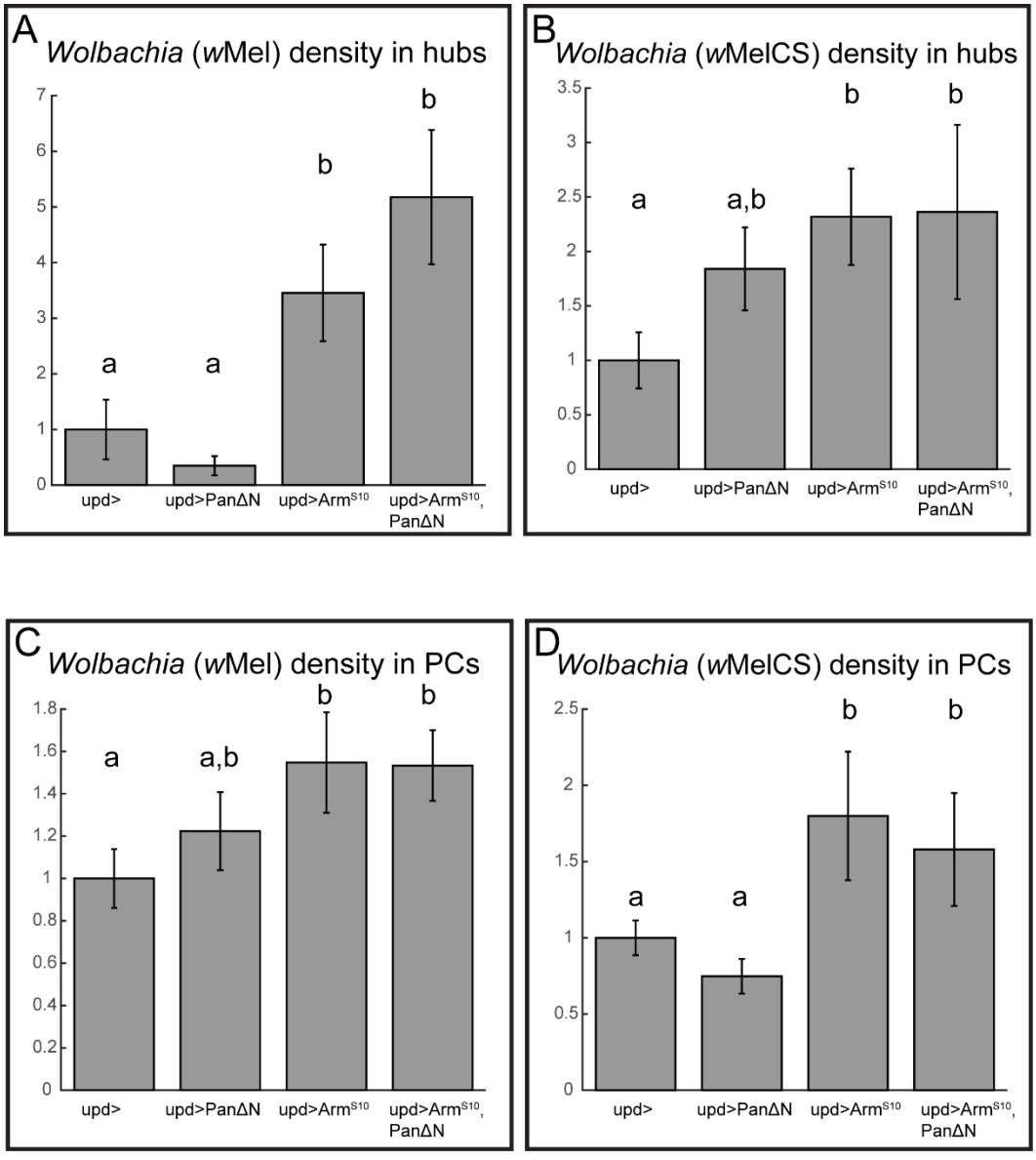
We expressed PanΔN in the hubs and PCs independently and epistatically with Arm^{S10} under the control of the *upd*-Gal4 driver. In the hubs, upon expression of Arm^{S10}, we see an increase in both *w*Mel (3.5-fold, Fig. D.1A) and *w*MelCS (2.4-fold, Fig. D.1B) levels. However, expression of both PanΔN and Arm^{S10} had similar increase in both *w*Mel (5-fold, Fig. D.1A) and *w*MelCS (2.4-fold, Fig. D.1B) levels. Although *w*Mel levels in both these treatments was different, the difference was not statistically significant.

Repeating the analysis with the PCs, we observe similar trends. Arm^{S10} expression caused an increase in *w*Mel (1.8-fold, Fig. D.1C) and *w*MelCS (1.6-fold, Fig. D.1D). expression of both PanΔN and Arm^{S10} together also increased *Wolbachia* density by similar magnitude (*w*Mel, 1.6-fold, Fig. D.1C; *w*MelCS, 1.6-fold, Fig. D.1D).

These findings suggest that Pangolin does not play a role in *Wolbachia* accumulation in the hubs and the PCs. Expression of PanΔN should have abolished the increase caused by Arm^{S10} in these cell types. Further analyses need to be performed to confirm whether the construct can knockdown Wnt signaling in these cell types. Moreover, we can knockdown other co-transcription factors required for this interaction. Hyrax (Hyx), Legless (Lgs) and Pygopus (Pygo) are required for Wnt target gene transcription along with Pan (Mosimann *et al.* 2006). Knocking down these proteins independently or epistatically with Arm^{S10} would provide further insights into the role of the transcription of Wnt target genes in *Wolbachia* accumulation. Alternately, Arm has been shown to interact with another transcription factor Sunspot (Ssp) to modulate gene expression (Taniue *et al.* 2010). The role of Ssp in *Wolbachia* accumulation can also be investigated.

Figure D.1 PanΔN does not rescue the increase in *Wolbachia* density caused by Arm^{S10}.

Quantification of *Wolbachia* density in hubs and polar cells (PCs) expressing Arm^{S10} and PanΔN shows that *Wolbachia* levels increase upon expression of Arm^{S10} but expression of PanΔN doesn't rescue the phenotype suggesting that Arm does not regulate *Wolbachia* density through Pan mediated transcription. (A) and (B) show *w*Mel and *w*MelCS density in hubs respectively. (C) and (D) show *w*Mel and *w*MelCS density in PCs respectively. a and b show statistically significantly distinct groups. Students' T test.


A
Wolbachia (*w*Mel) density in hubs
7
6
5
4
3
2
1
0
a
a
b
b
upd>
upd>PanΔN
upd>Arm^{S10}
upd>Arm^{S10}, PanΔN
B
Wolbachia (*w*MelCS) density in hubs
3.5
3
2.5
2
1.5
1
0.5
0
a
a,b
b
b
upd>
upd>PanΔN
upd>Arm^{S10}
upd>Arm^{S10}, PanΔN
C
Wolbachia (*w*Mel) density in PCs
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a,b
b
b
upd>
upd>PanΔN
upd>Arm^{S10}
upd>Arm^{S10}, PanΔN
D
Wolbachia (*w*MelCS) density in PCs
2.5
2
1.5
1
0.5
0
a
a
b
b
upd>
upd>PanΔN
upd>Arm^{S10}
upd>Arm^{S10}, PanΔN

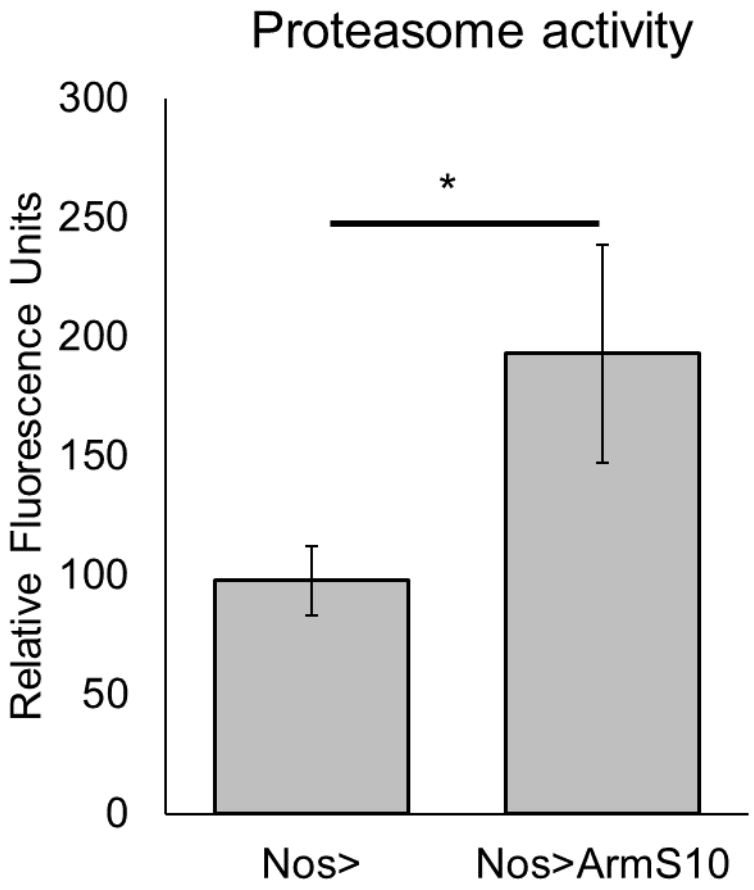
Appendix E: Active Wnt signaling leads to an increase in proteasome activity in whole ovaries

Amino acid availability is widely considered to be a mechanism that stimulates *Wolbachia* intracellular growth {Dunning Hotopp, 2006 #219;Wu, 2004 #220. High rates of proteasome mediated proteolysis would increase amino acid availability in *Drosophila* and mosquito cell lines (Fallon and Witthuhn 2009, White *et al.* 2017). Studies show that host proteasomal activity is increased in *Wolbachia* infection (Fallon and Witthuhn 2009, Zheng *et al.* 2011). Conversely, knockdown of proteasome activity in *Wolbachia* infected cells leads to reduction of infection (White *et al.* 2017). One of the mechanisms of Wnt mediated *Wolbachia* growth could be due to increased proteasomal activity.

To determine whether high Wnt signaling promotes high proteasome activity, we conducted a proteasome activity assay on ovaries expressing Arm^{S10} in the germline under the control of *nos*-Gal4 driver. Using a fluorogenic substrate, Z-LLE-AMC, we measured proteasome activity in protein lysates from whole ovaries (see chapter 2.8 for more details). Upon expression of Arm^{S10}, we observed a two-fold increase in proteasomal activity in the ovaries. This shows that activation of Wnt signaling leads to an increased proteasomal activity in *Drosophila* ovaries. This could potentially explain the increase in *Wolbachia* density upon Wnt activation.

Fig E.1 Proteasomal activity is increased upon activation of Wnt signaling

We measured Proteasomal activity in whole ovaries expressing Arm^{S10} under the control of *nos*-Gal4 using the fluorogenic substrate Z-LLE-AMC. Upon activation of Wnt signaling, we observed a two-fold increase in proteasomal activity. *p<0.05, Students' t test.



Appendix F: *Wolbachia* (*w*MelCS) infection partially rescues lethality induced by PanRNAi

To determine the effect of Pangolin (Pan) on *Wolbachia* accumulation, we also knocked it down in the hubs under the control of the *upd*-Gal4 driver. Pan acts as a repressor in absence of Wnt signaling {Archbold, 2014 #187;Schweizer, 2003 #192;Song, 2010 #281}. Thus, expressing *pan*RNAi under the control of *upd*-Gal4 led to partial lethality of the progeny. We believe this lethality is due to the expression of upd-Gal4 in the imaginal discs during pupal development.

The cross scheme and observed percentage of progeny is shown in Table F.1. We expected 33% of the progeny to be of the control genotype (CyO/ScO). In *w*- flies, we observed about 78% of progeny to be of the control genotype indicating there is a lethality induced by the expression of *pan*RNAi. In the *w*Mel infected flies, we observed similar percentage of control and experimental progeny. However, in *w*MelCS infected flies, we observed only 63% of the progeny to be of the control genotype. This suggests that *w*MelCS rescues the lethality of flies expressing *pan*RNAi. Chi squared tests were performed and these statistics are detailed in Table F.2.

Although preliminary, these findings show that *Wolbachia w*MelCS can rescue lethality induced by genetic aberrations in *Drosophila*. Further characterization needs to be done to determine whether this effect is reproducible for this and other genetic mutants.

Table F.1. Expected progeny from the *pan*RNAi cross

The parent cross setup to perform this experiment is shown here.

$$\frac{upd-Gal4}{upd-Gal4};\frac{CyO}{ScO};\frac{+}{+} \quad \text{X} \quad \frac{+}{-};\frac{CyO}{UAS-panRNAi};\frac{+}{+}$$

The table below shows the expected and observed ratio of F1 progeny from this cross.

Genotype	Expected	Observed		
		w-	*w*Mel	*w*MelCS
$\frac{upd-Gal4}{+};\frac{CyO}{ScO};\frac{+}{+}$	33%	76%	79%	63%
$\frac{upd-Gal4}{+};\frac{CyO}{UAS-panRNAi};\frac{+}{+}$	33%	8%	6%	14%
$\frac{upd-Gal4}{+};\frac{UAS-panRNAi}{ScO};\frac{+}{+}$	33%	16%	15%	23%

Table F.2. Chi-squared statistics

Chi squared statistics are calculated below. The critical chi-squared value for α=0.05 and df = 2 is 5.991. See table F.1 for the genotypes.

w- vs *w*Mel (Expected values are *w*- proportions)					
Genotype	Observed(O)	Expected(E)	(O-E)	(O-E)^2	((O-E)^2)/E
CyO/ScO (control)	227	218.78	8.24	67.81	0.31
*pan*RNAi/CyO	18	24.17	6.18	38.18	1.58
*pan*RNAi/ScO	14	46.05	2.06	4.22	0.09
TOTAL	289	289			$\chi 2 = 1.98$
w- vs *w*MelCS (Expected values are *w*- proportions)					
CyO/ScO (control)	166	201.35	-35.35	1249.62	6.21
*pan*RNAi/CyO	38	22.25	15.75	248	11.15
*pan*RNAi/ScO	62	42.4	19.6	384.16	9.06
TOTAL	266	266			**$\chi 2 = 26.42$**

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